PHENOTYPIC CONSEQUENCES OF IMPRINTING PERTURBATIONS AT *RASGRF1* IN MOUSE

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Nadia Meghann Drake

February 2010

PHENOTYPIC CONSEQUENCES OF IMPRINTING PERTURBATIONS AT *RASGRF1* IN MOUSE

Nadia Meghann Drake, Ph. D.

Cornell University 2010

Rasgrf1 is imprinted and paternally-expressed in neonatal mouse brain. At weaning, expression becomes biallelic. Manipulating the elements regulating imprinted *Rasgrf1* expression produces mice with transient perturbations in *Rasgrf1* imprinting, which we can use to assay the consequences of aberrant imprinting during a specific developmental time. As neonates, monoallelic-paternal (wild-type) mice paternally express *Rasgrf1*; biallelic mice express *Rasgrf1* from both alleles; null mice do not express *Rasgrf1*; and monoallelic-maternal mice reciprocally express *Rasgrf1*, from the maternal allele. This last genotype represents a unique opportunity to study the effects of expression derived from either of the two parental alleles. All genotypes biallelically express *Rasgrf1* around weaning.

Two phenotypes appear when *Rasgrf1* imprinted is perturbed: a difference in size, and a difference in performance on an associative odor-learning task at postnatal day 8 (P8). Size differences persist through adulthood and reflect the level of *Rasgrf1* expressed pre-weaning: biallelics are larger, nulls are smaller, and the two monoallelically-expressing genotypes are intermediate and indistinguishable from one another. We determined that neonatal *Rasgrf1* expression affects the functioning of the growth hormone/IGF-1 axis (GH/IGF-1 axis), with an input as far upstream as the

hypothalamus, and that *Rasgrf1* helps to set growth parameters early in development that persist through adulthood. Importantly, these data are the first experimental validation of one of the critical assumptions of the "conflict hypothesis," which describes the evolution of genomic imprinting in mammals.

Imprinting perturbations at *Rasgrf1* also produce performance differences on an associative odor-learning task in P8 neonates. The presence of a wild-type paternal allele is critical for proper task performance, as null mice, and those with maternally-derived *Rasgrf1* expression, do not perform as well. These differences in neonatal performance are accompanied by differences in *Rasgrf1* expression level and alterations in the amount of activated Ras and Rac protein in various brain structures. Differences in learning are not present in adult mice, suggesting that the role *Rasgrf1* plays in these phenotypes differs with respect to age, as well as the sensitivity of the system to *Rasgrf1* expression level.

BIOGRAPHICAL SKETCH

Nadia Drake was born on July 6, 1980 in Ithaca, New York to Frank and Amahl Drake. She moved with her family to Santa Cruz, California in 1984, which means that even though she is technically an Ithaca "townie" – she considers herself to be a Californian, adept at surviving earthquakes, fires, and merging into traffic.

In 1987, Nadia conducted her first science experiment, the protocol of which will be reproduced here:

Hypothesis: Birds can fly. They have feathers. *Ergo*, if Nadia had feathers, Nadia could fly too.

Materials: Feathers. Sweater (newer is better). Pine tree (taller is better).

Methods: Stick feathers through the arms of sweater. Do this until you think there are enough. Climb tree. Jump out. Flap arms. When you reach the clouds, bounce on them. Float gently down.

Result: a hard landing, and a sort of wrecked sweater.

Conclusion: This experiment was an epic failure. Suggestions for next time: improved feather:mass ratio. Padding.

Nadia continued doing backyard science "experiments" until she graduated as valedictorian of her Aptos High School class in 1998 (she even built a robot. Twice.). She then moved back to Ithaca to spend her undergraduate years at Cornell University. Upon entry, she was named a Meinig Family Cornell National Scholar, and proceeded to accumulate membership in various other organizations, including the Psi Chi National

Psychology Honor Society and the Mortarboard National Senior Honor Society, none of which meant much except for more decorations at graduation.

Nadia's academic 'experiments' in college were decidedly better documented than those in her early days: In 2000, she spent a summer doing research at Cornell University's Bourne Behavioral Neuroscience Lab (in White Plains, NY) studying appetite regulation and energy homeostasis using a rat model. In 2001, she taught for a summer at Yale University in a program aimed toward gifted high school students. She also completed a multidisciplinary senior research project investigating the various ways in which people watch and process dance performances, under the mentorship of Michael Spivey, Allen Fogelsanger, and Joyce Morgenroth. Her work combined data gathered using an "eye-tracker" with reported observations of dance to gain insight into how dance training affects dance viewing, on both macro- and micro-structural levels. Lastly, from 1998-2002, Nadia appeared to be working on answering the question: how many academic credits can a person accumulate in four years at Cornell? … The answer: 204. Of course, this experiment needs n>1, but subjects are hard to find, and the protocol would most likely not conform to acceptable ethical standards for the use of human subjects in research.

In addition, Nadia was also a member of the Cornell University Mock Trial team, and won various awards at regional and national levels for her contributions to the team. She also worked for the Santa Cruz County District Attorney for a bit (in 2000), where – in addition to case prep – she broke the

copy machine, fell asleep typing rejection letters, and was asked to perform a short ballet in the law library for the office's senior attorneys. No experiments were performed during this time, for the salient reason that to do so may lead to incarceration.

In 2002, Nadia graduated from Cornell University with a Bachelor of Arts in three different disciplines: biology (with a concentration in neuroscience), psychology, and dance.

After college, Nadia worked for two years (2002-2004) in a clinical cytogenetics lab at the Johns Hopkins University Medical School in Baltimore, Maryland. The lab focused on the prenatal diagnosis of chromosomal abnormalities, facilitated by karyotyping. Nadia can (still) tell you what the banding pattern of every human chromosome ought to look like, and furthermore, she completed an analysis of her own karyotype, where she discovered that alas – she has a stumpy chromosome 9, due to a polymorphic pericentric heterochromatic region ("I thought for sure yours would be long and elegant!" was her lab director's statement on the matter).

In 2004, Nadia returned to Cornell as a Presidential Genomics Fellow in Genetics & Development, and began to work with Dr. Paul Soloway. From 2004-2009, she studied how aberrant genomic imprinting contributes to various phenotypes in mouse, and conducted numerous experiments, the results of which will be reproduced in this document, in slightly more detail than the 1987 protocol above.

Other random facts and useless trivia:

1. Nadia has also spent much of her life dancing, since she began taking ballet classes at the age of 5. (a brief bio follows) Her very first role on stage was as a firefly, in 1986. When the Loma Prieta earthquake struck in 1989, Nadia was in ballet class – appropriately enough, her teacher had just instructed the pianist to "play something that moves!" In 1992, Nadia was awarded a full scholarship to study at the San Francisco Ballet School, and has since been offered admission to other nationally-recognized training programs, one of which she was almost kicked out of in the mid-nineties. In 1994, Nadia was selected as one of the most promising young dancers in the Western United States, but during 1995-1996, an injury prevented her from pursuing her dream of joining a professional company, so instead she took a trip to Europe for the summer. Nadia kept dancing, however, and in college was introduced to modern dance. She loved dancing at Cornell so much that she declared a dance major, and was the only person to graduate with a dance major in her year. While in Baltimore, Nadia danced with two companies -- the Mustard Seed Dance Company (modern dance), and the Baltimore Ballet – and since returning to Ithaca has been a principal dancer with the Ithaca Ballet. Nadia has danced many of the major roles in the classical ballet repertoire, as well as created many roles in new ballets and contemporary pieces through collaboration with various choreographers and artists. Nadia considers her participation in dance to be integral to her work in science, as they both require a considerable amount of creativity, dedication, and focus, and she considers herself incredibly fortunate to be afforded the opportunity to engage in science and dance simultaneously.

2. Nadia loves poetry.

3. Climbing trees is more fun in the dark.

4. Greek yogurt is Superfood.

5. Nadia fell off a chair and broke her arm at age 5.

6. Nadia enjoyed elementary school 'science fair' projects.

7. Nadia won the school spelling bee in 1991…and in 1993.

8. Nadia's permanent inhabitance of academic dork-dom occurred in 1992, when she asked her science teacher – loudly and conspicuously – if he gave A+'s. He said he did, but that no one had ever gotten one…

9. …Nadia got an A+ in 7th grade science class, and continuously corrected Mr. Cooper's spelling. I would've picked on her too, if I were a classmate.

10. One time, Nadia kicked herself in the head and fell over. The End.

إهداء

لأجلِ الحيوَانات.

لأجلِ تِلْكَ الرُوحِ اللتي تجَمَعُنا معاً.

لأجلِ السَّعْيِ وَراءَ المَعرِفَة، فَلتَكُن خفيفةً كما وهِيَ ثابِتَةٌ في وَطْنِها.

"وهَلْ مَعرِفَةُ الألفاظ إلاَّ ظلٌّ للمَعرفة بلا لَفْظٍ؟...وهـأنَذا قَد وَجَدتُ ما هوَ أعظمُ مِن الحِكمَة. إنَّها جَذْوةُ الروحِ فِيكُمُ تَتزَوّدُ دائِماً من ذاتِها، إنَّها لحياةٍ تَستَجدى حَيَاةً."

خَليل جُبران.

ACKNOWLEDGMENTS

"Those who bring sunshine to the lives of others cannot keep it from themselves"

James Matthew Barrie

"Gratitude is one of the least articulate of the emotions, especially when it is deep"

Felix Frankfurter

…And so, I would like to attempt to articulately express my deepest gratitude to those who have helped me to complete this chapter of my life, and acknowledge the sunshine they have brought to my days:

My advisor, Paul Soloway, has been an unbelievably tolerant and inspiring presence in my life over these five years. Not only is Paul an incredible scientist – one who is capable of expressing astounding intellectual curiosity and command, asking insightful questions, planning crazy experiments, and continuously disproving his own assertion that his brain is "like a sieve" – he is a remarkably caring and good-hearted person as well. I thank Paul for his scientific guidance, as well as his guidance along the many paths that life presents. He has been supportive and kind, patient and understanding, and willing to let me be who I needed to be, a meaningful and selfless contribution to this process, and the most that anyone could ever offer.

I thank the members of my committee – John Schimenti and Ruth Collins – for their guidance, support, and enthusiasm for my work. It has been a real privilege having the opportunity to interact with these two scientists, both of whom are exceptionally inquisitive and creative, and always generous with their time and input.

The Soloway Lab over the years has seen quite a few people come and go, so I will limit my discussion to those who have had the greatest impact on my time there. Rebecca Holmes – a warm, intelligent, and all-around awesome person – introduced me to the lab on my very first day as a rotation student, but her contributions have long outlived those early weeks, both personally and professionally. Jim Putnam – our generous and hard-working Mouse Wrangler – without whom I would have lost my mind much sooner than I actually did. Yoon Jung Park and Anders Lindroth – two of my absolute favorite people, who set a shining example with their hard work, persistence, and curiosity. Chelsea Brideau, who aptly demonstrates the powerful combination that is determination and intelligence. Patrick Murphy – who makes me laugh with his tales of intrigue – and reminds me why I love science in the first place. Ruqian Zhao was a selfless, helpful inspiration who believed in me when no one else did, and who helped me vanquish the western blot demons that had plagued me for many years. Her support, insight, and knowledge were essential to this process. And lastly, Al Armstrong – who for the first four years of my schooling was, in fact, the "Mouse King," and without whom I would have had mice coming out of my ears.

I want to thank those of my friends who have stuck by me through everything that life has thrown at me over these years, and provided countless instances of invaluable support and good times. Ed Strong – though an acquaintance since 1999 – burst into Ithaca in 2005 with his remarkable wit and promptly crashed into my low-hanging ceiling beams. Ed has been a true friend, unfailing in his support and ability to make me smile, and prompter of many fond memories involving such things as moldy pie, wine, Republicans,

and fun times with Mama and Papa (and Littlest) Drake. Ed is a treasure. Will Walker entered Cornell with me as a member of our class, and then entered my life as one of the most sensitive and understanding souls I have ever met. Will has been absolutely indispensable – one of those people with whom you can simultaneously share skydiving, Tokaji, and Yeats – and his presence has been similarly indispensable in helping me through graduate school and through life. In addition to those two, I have had the pleasure of interacting with many remarkable individuals over these years, though to individually acknowledge each would overwhelm this thesis, so naming will have to suffice: Kristy Tenny, Kellen Sensor, Nick Brideau, Katherine Kieckhafer, Claire Readhead, Janeen Streeter, Eric O'Brien (ninja!), Edoardo Carta-Gerardino, Jeff Jensen (and Winston and Molly), Samantha Dozier – you have all made my life richer and more meaningful.

During my time in graduate school, I also pursued my love of dancing, and was fortunate to have found a home at the Ithaca Ballet. I would like to acknowledge Deena Schmidt, my fellow principal dancer and partner in dancing/science-crime from the very beginning, for her friendship and encouragement. I will never forget our most enlightening dressing-room conversations! Johann Studier has been both an onstage partner and an offstage friend, supporting me in shoulder-sits and fish-dives as well as in life, and I thank him a million times for being as true a friend as one could ever, ever hope for. And I also thank him for undeniably delicious meals accompanied by boxed wine. Byron Suber – an absolute genius and gorgeous dancer – has been a shining presence in my life for over a decade. His beautiful ballets remind me why I love to dance, and I thank my lucky stars for these years with him. Lastly, Cindy Reid – director of the Ithaca Ballet and

a gorgeous dancer as well – has not only given me a stage on which to dance and a home in the studio, but the invaluable gift of her friendship. She has been, perhaps, my most called-upon source of support and has helped me to move through many dark times and emerge in the sunlight on the other side, in ways that no one else could. Cindy, Al, Pokie, and Yuliy – I thank you with all my heart.

On a different note, the six months leading up to the completion of this dissertation have been more challenging personally than I could have ever anticipated. I fell down a lot, but I was never allowed to fall all the way. Randy, Carolyn, and Anna – my triumvirate down the hill – have helped me to stay healthy and whole, and they pick me up when I can't do it alone. Clare, Amy, and Bruce – my triumvirate down the road – helped to glue the pieces of myself back together when I needed it the most, to call upon the energies within me to help me move forward, and to remember the value of forming meaningful personal connections. All of their support has meant more to me than I could ever express in words.

Finally, my family…has given me more than I could have ever hoped or even thought to ask for. From the day I was born I was encouraged to follow my dreams, to think big, and to be fearless in pursuing my heart's desires. My parents are my heroes, and set wonderful examples for me to follow. They take wonderful care of me, and their unconditional love and support mean the world to me. My sister is the brightest ray of sunshine I have ever encountered (she puts the sun to shame), the best person I know, and my best friend. Growing up, she and I were taught the value of a proper education and encouraged to let our imaginations run wild (which resulted in such things as backyard "science" experiments, attempts to inconspicuously

read books during dinner, and countless hours of taxi service to ballet classes, auditions, and performances), and we are where we are today because of the love our parents gave us. I also look to the examples set by my grandparents, who – though they are no longer with us – live on in my daily thoughts and in the light of the moon. I was fortunate ('blessed,' if you will) to be born into a most amazing and delightful family, with generations of solid examples to follow.

Lastly, my little Ithaca family has been of paramount significance in the completion of this dissertation because without them, I quite literally would not be here. Kaleb and Posie – my two four-legged fur children – have kept me safe, warm, and loved. They give generously with all of their being and remind me that life is worth living, even if just for one more day of Posie-kisses, Kaleb-smiles, and doggie-piles (in which I am most inevitably at the bottom). Their faces are more familiar to me than my own, and I only hope that I reflect back at them what they bestow so brightly upon me.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

APPENDICES

LIST OF TABLES

CHAPTER I

LIST OF ABBREVIATIONS

A(vy) – agouti viable yellow allele

ABI – Applied Biosystems

ALS – acid-labile subunit

AMPA – a-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

AS – Angelman Syndrome

B – biallelic

bp – base pairs

BWS – Beckwith-Wiedemann Syndrome

C – cut

CDC25 – cell division cycle 25 homolog

cDNA – complementary DNA

cm – centimeter

CpG – cytosine followed by a guanine

CpNpG – cytosine followed by any base, then a guanine

CpNpN – cytosine followed by any two bases

CREB – camp response element binding

CTCF – CCCTC binding factor

DH/PH – Dbl-homology/Pleckstrin homology

DMD – differentially methylated domain

DNA – deoxyribonucleic acid

DNMT – DNA methyltransferase

EDTA – ethylenediaminetetraacetic acid

ENU – n-ethyl-n-nitrosourea

ERK – extracellular signal-regulated kinase

Fig. – figure

FMK – furyl methyl ketone

FVB/n – FVB/NJ

GAP – GTPase activating protein

GDP/GTP – guanosine diphosphate/guanosine triphosphate

GEF – guanine nucleotide exchange factor

GH – growth hormone

GH-R – growth hormone receptor

GHRH – growth hormone releasing hormone

GHRH-R – growth hormone releasing hormone receptor

GluR1 – AMPA receptor subtype, GluR1

H2A, H2B, H3, H4 – histone subunits

H3K27me3 – trimethylated histone 3, residue lysine 27

H3K4me3 – trimethylated histone 3, residue lysine 4

H3K9me3 – trimethylated histone 3, residue lysine 9

H4K20me3 – trimethylated histone 4, residue lysine 20

H4K36me3 – trimethylated histone 4, residue lysine 36

HA – n-hexyl acetate

HBSS – Hanks' Balanced Salt Solution

HDAC – histone deacetylase

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP – horseradish peroxidase

IAP – intracisternal A particle

ICR – imprinting control region

IGF-1 – insulin-like growth factor 1

IGF1-R – IGF-1 receptor

IGFBP – IGF1 binding protein

IgG – immunoglobulin G

IHC -- immunohistochemistry

IQ -- ilimaquinone

Kb -- kilobase

KCl – potassium chloride (salt you should not eat)

kD – kilo Dalton

LPA – lysophosphatidic acid

LTD – long-term depression

LTP – long-term potentiation

MANOVA – multiple repeated measures analysis of variance

MAP2 – microtubule-associated protein

MAPK – mitogen-activated protein kinase

MBD – methyl binding domain

mM – millimolar

MM – monoallelic maternal

MP – monoallelic paternal

mRNA – messenger RNA

N – null

ncRNA – non-coding RNA

NMDA – n-methyl-d-aspartic acid

NMR – nuclear magnetic resonance

NR2B – NMDA receptor subunit NR2B

NSE – neuron-specific enolase

OTB – ovarian time bomb

P(number) – indicates post-natal day (number); ie, P8 = postnatal day 8

Pa – Pascals

PCR – polymerase chain reaction

PGK – phosphoglycerate kinase

PKA – protein kinase A

PWK – PWK/PhJ

PWS – Prader-Willi Syndrome

qPCR/qRT-PCR – quantitative PCR/reverse transcriptase PCR

REM – Ras-exchanger motif domain

RNA – ribonucleic acid

rRNA – ribonuclear RNA

RT-PCR – reverse transcriptase PCR

SCBT – Santa Cruz Biotechnology

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

UC – uncut

ul – microliter

uM – micromolar

UTR -- untranslated region

wt – wild-type

♀ female

♂ male

\+ wild-type

\- null

ß beta

I. INTRODUCTION

I.1. Epigenetics

I.1.1 Definition

In 1942, Conrad Waddington described the result of genes interacting with their surroundings as an "epigenetic landscape" – a metaphor for processes occurring during developmental cellular differentiation (Waddington, 1942). Originally, "epigenetics" was simply a portmanteau comprised of "genetics" and "epigenesis," and was defined as the process by which genotypes produce phenotypes during development. In the intervening years, the definition of "epigenetics" has broadened to encompass biological processes unrelated to development, as well as narrowed to focus on non-mutational, heritable modifications that produce variant phenotypes.

Today, "epigenetics" refers to mechanisms that affect gene expression independently of nucleotide mutations (Russo *et al.*, 1996), meaning that mutant phenotypes can be produced in the absence of sequence variation. The mechanisms producing these changes in expression are stable and heritable, yet reversible, and can be influenced by environmental factors such as diet, maternal behavior, and toxins (Waterland and Jirtle, 2003; Weaver *et al.*, 2004; Anway *et al.*, 2005), in addition to endogenous biological catalysts. The consequences of epigenetic changes can persist through multiple rounds of cell division, with the best-known example being the differentiation of totipotent stem cells into pluripotent cell lines during normal embryonic

development, since differentiation involves cells that are genetically identical but epigenetically distinct (Bernstein *et al.,* 2006). On an organismal level, the phenotypic consequences of epigenetic alterations can be penetrant through multiple subsequent generations (reviewed in Jablonka and Raz, 2009; Anway *et al.,* 2005; Rakyan *et al.,* 2003); phenotypes are then available for selection to act upon, yet such epigenetic changes do not leave a detectable signature at the sequence level, which suggests that such modifications may play a significant – but difficult to detect – role in shaping evolutionary processes.

Epigenetic modifications exist in a number of organisms, including fission yeast (*Schizosaccharomyces pombe*; Grewal and Klar, 1996), flowering plants (*Arabidopsis thaliana*; Vongs *et al.,* 1993), invertebrates (*Drosophila melanogaster*; Cleard *et al.,* 1997), and mammals (*Mus musculus, Homo sapiens*; Li *et al.,* 1993; Fraga *et al.,* 2005). Epigenetic mechanisms are essential for a number of biological processes including cellular differentiation (reviewed in Reik, 2007), paramutation (reviewed in Chandler, 2007), X-chromosome inactivation (Panning & Jaenisch, 1996), genomic imprinting (Stoger *et al.*, 1993; Ferguson-Smith *et al.*, 1993), transcription, and chromosome dynamics (reviewed in Goll & Bestor, 2005). Additionally, such modifications are implicated in the production of a number of pathological states in humans, including cancer, mental disorders, metabolic processes, and conditions associated with aberrant genomic imprinting (ie, Prader-Willi and Angelman syndromes; reviewed in Jaenisch & Bird, 2003).

I.1.2 Modifications & Mechanisms

Epigenetic modifications include the covalent attachment of various chemical groups to DNA itself or to the histone proteins around which DNA is packaged.

I.1.2.1 DNA Methylation

In mammals, most DNA is methylated by the covalent attachment of a methyl group to the 5′ carbon of a cytosine residue that occurs as part of a CpG dinucleotide. CpG dinucleotides are symmetric and can cluster to form "islands," which are sequences of DNA rich in CG residues as well as CpG dinucleotides. "Hemimethylated" DNA exists when one strand of DNA is methylated, and the other is unmethylated; the methylated strand can serve as a template for the proper placement of methyl groups on the other strand – during DNA replication, for example. DNA methylation is observed at CpG islands, which are often present near promoters; methylation is also observed within repetitive regions like satellite sequences and transposable elements, and centromeres. Hydroxymethylation of cytosine residues has recently been reported to exist in mammalian neurons (Kriaucionis and Heintz, 2009), though little is known about its distribution outside the brain. DNA methylation has also been observed and well-characterized in flowering plants, though the pattern differs from that observed in mammals: in addition to CpG dinucleotides, (symmetric) CpNpG and (asymmetric) CpNpN sequences are also targets for methylation (Zilberman *et al.*, 2007). DNA methylation has been identified at low levels in *Drosophila* and *Neurospora*, though has not been detected in yeast or worm (Bull and Wootton, 1984;

reviewed in Bird, 2002). Additionally, non-CpG methylation has been reported in mammals, with nearly one-quarter of methylation in human embryonic stem cells present in a non-CpG context – though differentiation of stem cells leads to the disappearance of non-CpG methylation (Lister *et al.*, 2009).

DNA methylation is catalyzed by a class of enzymes known as DNA methyltransferases (DNMTs), which transfer a methyl group from the methyl donor S-adenosylmethionine to the cytosine residue. Several DNMTs have been characterized in mammals: *Dnmt1*, *Dnmt3a*, and *Dnmt3b*. Mammalian *Dnmt1* mutants are embryonic lethal (Li *et al.,* 1992), pointing to an important role for DNA methylation in normal growth and development. *Dnmt1* is a maintenance methyltransferase, meaning that it catalyzes reactions at hemimethylated loci, while *Dnmt3a* and *3b* are *de novo* methyltransferases, capable of methylating a completely unmethylated strand of DNA. The conversion of 5-methylcytosine to 5-hydroxymethylcytosine can be accomplished by the enzyme TET1, though this relationship has only been demonstrated in cell culture and *in vitro* (Tahiliani *et al.*, 2009). The enzyme(s) supporting the opposite reaction – the removal of a methyl group – has not been well-characterized in vertebrates, but evidence for mechanisms exists. In *Xenopus laevis* oocytes, *Gadd45a* and the DNA repair endonuclease XPG are involved in DNA repair-mediated demethylation that activates methylation-silenced reporter plasmids (Barreto *et al.*, 2007). In zebrafish embryos, injection of methylated DNA induces active demethylation, which is mediated by a combination of Gadd45, a 5-methylcytosine deaminase (AID), and a G:T mismatch-specific thymine glycosylase (Mbd4; Rai *et al.,* 2008). In mouse,

Gadd45b-mediated demethylation has been demonstrated to occur in a neuronal activity-dependent fashion in mature hippocampal neurons, which links neural activity and processes involved in plasticity with epigenetic remodeling (Ma *et al.*, 2009). Demethylating enzymes have been identified and characterized in *Arabidopsis*, and these enzymes – DME and ROS1 – act as DNA glycosylases and apurinic/apyrimidinic lyases(Gehring *et al.*, 2006; Gong *et al.*, 2006). Demethylation is an active and essential process during mammalian development (Oswald *et al.,* 2000; reviewed in Morgan *et al.,* 2005).

I.1.2.2 Histone Modifications

Histone proteins are targets for epigenetic marking, and modifications affect higher-order chromatin structure and the transcriptional accessibility of a locus. Histones form an octamer comprised of four different subunits in duplicate (H2A, H2B, H3, and H4), and when DNA is coiled around these octamers the structure is known as a nucleosome; nucleosomes are then organized and condensed to form chromosomes. The amount of condensation can vary: DNA supported by loosely packaged stretches of nucleosomes is more transcriptionally accessible, while tightly coiled regions are more difficult for transcriptional machinery to access. Epigenetic modifications to histone proteins work to produce these changes in chromatin structure by the recruitment of chromatin remodeling factors and complexes, which work to facilitate higher–order regulation of gene transcription (reviewed in Knoepfler and Eisenman, 1999; Schulze & Wallrath, 2007).

Commonly, histones are post-translationally modified at specific amino acids in their N-terminal tails, which project outward from the core of the nucleosome. Modifiers are comprised of a broader class of chemical groups: in addition to methylation, histones can be acetylated, phosphorylated, ubiquitinated, ribosylated, and sumolated. Chromatin conformation is regionally affected by modifications to histone proteins: an abundance of silencing or activating marks in an area can create either a transcriptionally-repressed (closed) chromatin conformation, or a transcriptionally-active (open) conformation. Not surprisingly, the predominance of these marks varies along the length of a chromosome, with centromeric and condensed (heterochromatic) regions being characterized by a set of marks that differs from those found in less tightly-packed (euchromatic) areas. Additionally, marks can exist in different patterns during development, and different distributions of histone modifications are coincident with processes like cellular differentiation (Bernstein *et al.*, 2006), and can mark different cell lineages (Mikkelsen *et al.*, 2007).

I.1.3 Effects of Epigenetic Marks on Transcription

The specific effect of a modification on expression cannot readily be predicted by the type of modification present. Rather, the location of the modification within either the DNA sequence or at a specific histone residue determines whether the mark will act to silence or promote gene expression; in some cases, even the number of groups attached has significant functional importance.

For example, methyl groups placed on DNA close to a gene's promoter may act as repressive elements – indeed, DNA methylation is thought to have evolved as a mechanism for silencing transposable elements and foreign DNA insertions (reviewed in Slotkin & Martienssen, 2007). DNA methylation at gene promoters can recruit methylated-CpG binding proteins (MBDs), which in turn can recruit corepressors, remodeling complexes, and histone deacetylases (HDACs), which all act in concert to create a repressive chromatin conformation at a locus (Boyes & Bird, 1991; Nan *et al.,* 1998; Dobosy & Selker, 2001).

However, methyl groups placed within a regulatory sequence can facilitate transcription of a gene, a pattern observed at the *Rasgrf1* locus in mouse (Yoon *et al.,* 2002; Yoon *et al.,* 2005). At this locus, DNA methylation prevents the binding of CCCTC binding factor (CTCF), a methylation-sensitive enhancer-blocking protein that is highly conserved among taxa. When CTCF is bound to a sequence, it can block promoter-enhancer interaction and thus leads to transcriptional silencing. A second major function of CTCF in the genome is to act as an insulator, preventing the spread of heterochromatin (Bell *et al.,* 1999; reviewed in Ohlsson *et al.,* 2001).

Modifier groups placed on histone proteins have different effects depending both on the placement and the number of marks present; modification of lysine residues has been the most extensively profiled, with acetylation generally acting to increase transcriptional accessibility of a locus, and methylation having varied effects on expression. For example, 'silencing'

marks include trimethylated lysine 9 on histone H3 (H3K9me3), trimethylated lysine 20 on histone H4 (H4K20me3), and trimethylated lysine 27 on histone H3 (H3K27me3), and 'activating' marks include trimethylated lysine 4 on histone H3 (H3K4me3) and trimethylated lysine 36 on histone H4 (H4K36me3); additionally, monomethylated H3K9 is associated with transcribed regions, meaning that even the number of methyl marks placed can have different effects (Peterson & Laniel, 2004; Li *et al.*, 2007; Barski *et al.*, 2007). Often, marks compete with one another for placement and overall effect on transcription within a certain region, an observation that has led to the development of the 'histone code' which identifies histone modifications as mediating the interaction between chromatin, DNA, and transcriptional status (Jenuwein & Allis 2001). Also of importance is the hierarchical relationship between the placement of DNA methylation and histone modifications, with the suggestion of mutual exclusion and antagonism existing for some types of marks: for example, DNA methylation and H3K27 trimethylation (H3K27me3) cannot exist at the same sequence at the *Rasgrf1* locus, as placement of one mark prevents placement of the other mark (Lindroth *et al.*, 2008).

I.1.4 Environmental Inputs and Disease States

Epigenetic mechanisms confer a certain amount of genomic flexibility in that they can be inherited in non-Mendelian ratios, are sensitive to a variety of environmental inputs, and reversibly affect gene expression. Indeed, such mechanisms are postulated to form one of the links connecting gene

expression with environmental contributions. However, aberrant placement of epigenetic marks can lead to disease states – in model organisms as well as in humans – and the placement of these marks can persist through multiple generations, affecting the fitness of individuals in the absence of the initial stimulus.

Diet and toxins have been observed to significantly and transgenerationally affect epigenetic modifications. For example, gestating rats transiently exposed to the fungicide vinclozolin produce an F1 generation with reported genome-wide changes in DNA methylation, and aberrant phenotypes – which include increased rates of male infertility and a decrease in spermatogenic capacity – are observed four generations post-exposure (Anway *et al.*, 2005). Feeding a methyl-supplemented diet to gestating viable yellow agouti (A(vy)) mice leads to DNA methylation at the A(vy) locus (termed a 'metastable epiallele') in progeny, which persists through adulthood and produces phenotypic alterations (Waterland & Jirtle, 2003). Similar effects are observed at the Axin-Fused (Axin(Fu)) locus in mouse, another metastable epiallele, with methyl-supplemented maternal diets producing aberrant patterns of DNA methylation (and altered phenotypes) in progeny (Waterland *et al.*, 2006). Both the A(vy) and Axin(fu) loci contain a retrotransposon-derived intracisternal A particle (IAP), which may make these loci especially susceptible to diet-induced changes in methylation. Pregnant rats exposed to a low-protein diet produced progeny with reduced DNA methylation at specific loci; supplementing that low-protein diet with folic acid prevented alterations in DNA methylation (Lillycrop *et al.*, 2005), which otherwise persisted through adulthood (Lillycrop *et al.*, 2008). Additionally, the

incidence of mortality due to cardiovascular disease and diabetes has been epidemiologically shown to correlate with both parental and grandparental nutrition state in humans (Kaati *et al.*, 2002), suggesting that a mechanism not unlike those observed in rodents might be affecting the expression of genes responsible for producing these disease states.

Evidence in rats suggests that maternal behavior can affect the epigenetic state of sensitive loci in offspring. Variability in the grooming styles of female rats was demonstrated to affect DNA methylation, histone acetylation, and transcription factor binding at the glucocorticoid receptor in progeny (Weaver *et al.*, 2004). Exposure later in life to a diet high in methyl-donors reversed the early effects of maternal behavior on the epigenome (Weaver *et al.*, 2005), suggesting again that such epigenetic modifications are sensitive to environmental inputs and confer a higher level of genomic plasticity than sequence alone is capable of.

The aberrant placement of epigenetic marks produces diseases in humans as well as in model organisms. Perhaps the most-studied example of such processes is cancer, with the first demonstration of a link between DNA methylation and cancer occurring over two decades ago (Feinberg & Vogelstein, 1983). More recently, the role of epigenetic processes in cognitive function and disorder is beginning to be elucidated. Epigenetic factors are postulated to play a part in the presentation of such conditions as Alzheimer's disease and Rett syndrome (reviewed in Graff & Mansuy, 2009), a variety of mental retardation disorders (reviewed in Urdinguio *et al.*, 2009), drug addiction and depression (reviewed in Renthal & Nestler, 2009), schizophrenia

(Roth *et al.*, 2009), and post-traumatic stress disorder (Yehuda & Bierer, 2009), suggesting that the phenotypic effects of epigenetic marks are present in organisms at all levels, from the beginning of embryonic development to neurodegeneration in later life.

I.2. Genomic Imprinting

Genomic imprinting in mammals refers to the proscribed allele-specific expression of a gene, with transcriptional activity determined by the parent from which an allele is inherited. Some genes are only expressed from the maternal allele – with corresponding silencing of the paternal allele – and some are expressed in the opposite pattern (reviewed in Delaval & Feil, 2004; Wilkins & Haig, 2003). Imprinting exists at a small subset of mammalian genes – to date, there are only around 100 imprinted genes identified in mouse, though a larger number are predicted to be imprinted based on sequence features (Luedi *et al.*, 2007), and still more predicted, and validated, based on the clustering of various epigenetic marks (Brideau *et al.,* submitted, 2009). Imprinting has been identified in mammals, marsupials, and plants (Weidman *et al.*,2004; Suzuki *et al.*,2005; Edwards *et al.,* 2007, Martienssen *et al.*, 2008), with suggestions of imprinting-like processes in social insects (Queller, 2003; Kronauer, 2008; Elango *et al.,* 2009).

Imprinting at a locus can be more complicated than monoallelic transcription in all tissues at all ages. In some cases, imprinted expression is restricted to a specific type of tissue or developmental stage. In the case of *Igf2r*, for example, maternal-specific expression occurs in non-neuronal cells

within the mouse brain and other somatic tissues throughout the organism, but in neurons, expression is biallelic (Barlow *et al.*, 1991; Hu *et al.*, 1998; Yamasaki *et al.*, 2005). In mouse, *Ube3a* is maternally-expressed in neurons but biallelically-expressed in other somatic tissues (Yamasaki *et al.*, 2003), while *Grb10* is predominantly paternally-expressed in brain, but maternally-expressed in other somatic tissues (Hikichi *et al.*, 2003). Additionally, imprinting at a locus may vary with developmental stage: *Murr1* is imprinted in adult mouse brain, but biallelically expressed in embryonic and neonatal brain tissue (Wang *et al.*, 2004), while *Rasgrf1* is paternally-expressed in neonatal mouse brain, but gradually transitions to biallelic expression around the time of weaning (Plass *et al.*, 1996, Drake *et al.*, 2009). Other loci are only imprinted in the placenta (reviewed in Wagschal & Feil, 2006).

I.2.1 Epigenetic Regulation of Imprinting

Allele-specific epigenetic patterns accompany the allele-specific expression characteristic of imprinting (Mager & Bartolomei, 2005; Edwards & Ferguson-Smith, 2007). Placement of these marks occurs in the germline, and the developing organism retains these parental marks in somatic tissues, yet reprograms its germline to reflect its own sex (reviewed in Santos & Dean, 2004; Morgan, 2005; Reik, 2007). Reprogramming and the mechanisms that regulate it are being extensively studied, as similar mechanisms may be responsible for the aberrant placement of epigenetic marks that result in disease states associated with misimprinting, as well as in the development of cancer cells and the regulation of their growth and immortality (Robertson, 2005).

I.2.1.1 DNA methylation

Allele-specific methylation patterns are observed at the imprinting control regions (ICRs) that govern imprinted expression, and in mouse, all but three loci (*Rasgrf1*, *H19*/*Igf2*, *Dlk1*/*Gtl1*) exhibit maternal-allele specific methylation. Imprinted genes tend to occur in clusters throughout the genome, and one ICR can affect the imprinted expression of multiple genes (reviewed in Reik & Walter, 2001). Methylation can act to impart imprinted expression by affecting the activity of enhancer-blocking elements (at the *H19*/*Igf2* locus, Bell & Felsenfeld, 2000; at the *Rasgrf1* locus, Yoon *et al.*, 2005), the transcription of antisense non-coding RNAs (ncRNAs; *Igf2r*/*Air* transcript, Sleutels *et al.*, 2002; *Kcnq1*/*Kcnq1ot1*, Mancini-Dinardo *et al.*, 2006), and the placement of histone modifications (Delaval *et al.*, 2007; Lindroth *et al.*, 2008). Further evidence for the importance of DNA methylation in imparting proper imprinted expression is derived from loss-of-function *Dnmt1* mutant mouse embryos, which exhibit a disruption in normal imprinted expression patterns at the *H19*, *Igf2*, and *Igf2r* loci (Li *et al.*, 1993).

I.2.1.2 Histone modifications and RNA

Differential histone modifications are also associated with both the expressed and silent alleles for several imprinted loci studied, though are not as well-characterized as DNA methylation in the imprinting process. However, there is ample evidence that epigenetic tags differ with parental allele, and that these tags are correlated with expression pattern (Delaval *et al.*, 2007; Lindroth *et al.*, 2008). Additionally, imprinting can result from the

production of ncRNAs that direct the silencing of a particular allele, as is the case with the *Igf2r*/*Air* and *Kcnq1*/*Kcnq1ot1* loci. Both of these loci contain ncRNA promoters within their ICRs, and methylation of the ICR prevents transcription of the ncRNA (Wutz *et al.*, 1997; Thakur *et al.*, 2004). Thus while the mechanisms regulating genomic imprinting in mammals are becoming better understood, the function of imprinted expression is still a fundamental question.

I.2.2 The Evolution of Imprinting: the conflict hypothesis

One might question the selective advantage of forgoing diploidy at an imprinted locus: indeed, silencing one of two alleles present could be detrimental in a case where the expressed allele contains a recessive mutation. In this situation, despite the presence of a wild-type allele, expression will only be derived from the mutated allele, which could lead to reduced fitness of the organism. Diseases associated with imprinting mutations exist in humans and can be modeled in other organisms. Beckwith-Wiedemann syndrome (BWS), Prader-Willi syndrome (PWS), and Angelman Syndrome (AS) occur when imprinted genes are improperly expressed (reviewed in Robertson, 2005). [Alarmingly, the incidence of imprinting-related diseases is increasing, especially among children conceived through the use of assisted reproductive technologies, which suggests that the processes necessary for maintaining proper epigenetic marking may be adversely affected by early embryo manipulations (Cox *et al.*, 2002; Maher *et al.*, 2003).]

Thus one fundamental question in the study of genomic imprinting is how imprinting evolved in mammals, despite the consequences of giving up diploid protection against recessive mutations.

The theory explaining genomic imprinting in mammals that is best supported by experimental evidence is known as the "conflict hypothesis" or the "kinship hypothesis". Proposed by Moore and Haig in 1991 (Moore & Haig, 1991), the conflict hypothesis suggests that imprinting results from the differing interests of the maternal and paternal genomes with regard to maternal resource allocation and offspring development. In non-monogamous species – where paternity is not a foregone conclusion – it is in the paternal genome's interest to maximize maternal resource distribution to pups in one litter – the litter that is most likely sired by a given father. Thus, in an effort to maximize reproductive fitness, the paternal genome acts to promote maternal resource distribution to pups of known paternity, even to the potential detriment of both the mother and pups sired by other fathers. The maternal genome, however, is more conservative when considering how resources ought to be distributed and instead maximizes reproductive fitness by more parsimoniously allocating resources, both among pups within a single litter, across litters, and the mother herself. It is more costly and difficult for the maternal genome to reproduce, thus producing the most progeny with the greatest chance of survival is a priority.

One prediction of the conflict hypothesis is therefore that paternally-expressed genes will act to maximize the growth of offspring in such a way that demand for maternal resources is greatest; in other words, paternally-

expressed genes are predicted to act as growth-promoters. On the contrary, maternally-expressed genes are predicted to inhibit offspring growth so that the development of future litters and the maintenance of maternal reproductive fitness can be maximized. Indeed, there are numerous examples of paternally-expressed genes acting as offspring growth promoters (*Snrpn*, *Peg3*, *Peg1*, *Igf2*, *Rasgrf1*) and maternally-expressed genes acting as offspring growth inhibitors (*Ube3a*, *Gnas*, *Grb10*, *Igf2r*). One elegant example in mouse is the *Igf2*/*Igf2r* relationship: *Igf2* is a paternally-expressed, growth promoting gene, and loss of *Igf2* expression leads to intrauterine growth retardation (DeChiara *et al.*, 1990). *Igf2r* is materally-expressed, acts as a growth inhibitor, and codes for the IGF2 receptor; loss of *Igf2r* expression leads to fetal overgrowth (Ludwig *et al.*, 1996). The mechanism whereby expression at imprinted loci becomes preferentially monoallelic is proposed to resemble a gradual tug-of-war between the genomes, which results ultimately in monoallelic expression from one genome or the other as gradual increases or decreases in expression are modulated (Wilkins & Haig, 2003).

I.2.2.1 A critical assumption

A fundamental assumption that this theory rests on is that gene expression level is the critical piece in contention (Wilkins & Haig, 2003), and that monoallelic expression of equivalent quantity from one genome or the other does not produce a fundamentally different phenotype. The conflict hypothesis assumes that for each imprinted gene, there is a maximally beneficial level of gene expression (for one genome or the other), and that the evolutionary tug of war is engaged in to produce this optimum level. In the

case of genes that promote offspring growth, this leads to silencing of the maternal allele in response to increased levels of expression from the paternal allele, and vice versa in the case of genes inhibiting offspring growth. To date, however, while there are many instances where individual gene expression patterns tend to support the conflict hypothesis, there has only been one study done which tests this fundamental assumption (Drake *et al.*, 2009). However, it is has been proposed that loci where recessive mutations might produce a deleterious phenotype are the least likely to be imprinted (Ubeda & Wilkins, 2008).

I.2.2.2 Additional theories

Other theories do exist to explain the development of mammalian imprinting, yet experimental evidence for these is not as strong.

One early theory suggested that genomic imprinting can lead to reproductive isolation and hence evolved as a post-zygotic speciation mechanism (Varmuza, 1991).

'Evolvability' models propose that haploidy at imprinted loci promotes enhanced adaptability within a population by 'hiding' the silenced alleles from natural selection (Beaudet & Jiang, 2002). In this scenario, those silent alleles are afforded the opportunity to accumulate multiple potentially deleterious mutations that could interact synergistically with other similar loci to produce an adaptive phenotype; these same mutations would otherwise be eliminated if they were immediately visible to natural selection. Thus the evolvability hypothesis suggests imprinting contributes to the development of a pool of

hidden genetic variation, but does not provide an explanation for which loci are imprinted, or which parental allele is silenced.

The 'ovarian time bomb' hypothesis (OTB) suggests that imprinting evolved to prevent ovarian teratomas from producing invasive trophoblasts (Varmuza & Mann, 1994), since paternally-contributed genetic material is necessary for trophoblast development. Thus, the genes required for trophoblast development are predicted to be maternally-silenced and paternally-expressed, which is the pattern observed. However, the OTB fails to explain why we see imprinting at loci unimportant for trophoblast development, why imprinting persists in somatic tissues, why some paternal genes are silenced, and why imprinting exists in species lacking invasive placentas.

The 'maternal-offspring' coadaptation theory (Wolf & Hager, 2006) suggests that imprinting increases the adaptive integration of maternal and offspring genomes, which leads to higher fitness for the offspring, facilitates beneficial maternal-offspring coadaptive trait development, and explains the relative overabundance of maternally-expressed autosomal genes (particularly in the placenta).

Collectively, these theories fail to address various characteristics of imprinted loci, including which parental allele is silenced, why certain loci are imprinted, the presence of imprinted expression in somatic tissues, and the presence of imprinting in species lacking invasive placentas.

I.3. *Rasgrf1*

I.3.1 Imprinting

Imprinting at *Rasgrf1* is controlled by the placement of methylation at a differentially methylated domain (DMD) located 30kb upstream of the gene's promoter (Yoon *et al.*, 2002; **Figure I.1**). The locus contains two elements that impart imprinted expression: the DMD, which is capable of being methylated, and a series of repeats which immediately flank the DMD and act to direct methylation. On the transcribed paternal allele, the DMD is methylated; on the silenced maternal allele, the DMD is unmethylated.

The placement of methylation is directed by a signal emanating from the repeats; the repeat unit consists of a 41-mer and is repeated 40 times. Excision of the repeats (the *tm1* allele; **Figure I.2**) leads to a failure to acquire methylation at the DMD, which in turns leads to allele silencing. Silencing at the maternal allele is due to the presence of CTCF – a methylation-sensitive enhancer-blocking protein, which can bind the DMD in the absence of methylation (Yoon *et al.*, 2005). CTCF blocks communication between a presumed endogenous enhancer (not yet identified) and the *Rasgrf1* promoter; when the DMD is methylated, CTCF cannot bind, and the enhancer can act in concert with the promoter to produce gene expression. Replacement of the repeats with the PGK enhancer (the *tm2* allele; **Figure I.2**) leads to allele transcription, suggesting that the placement of an enhancer between the CTCF-bound DMD and promoter is sufficient to activate transcription. The mechanism whereby the maternal allele becomes active and escapes silencing around weaning is unknown.

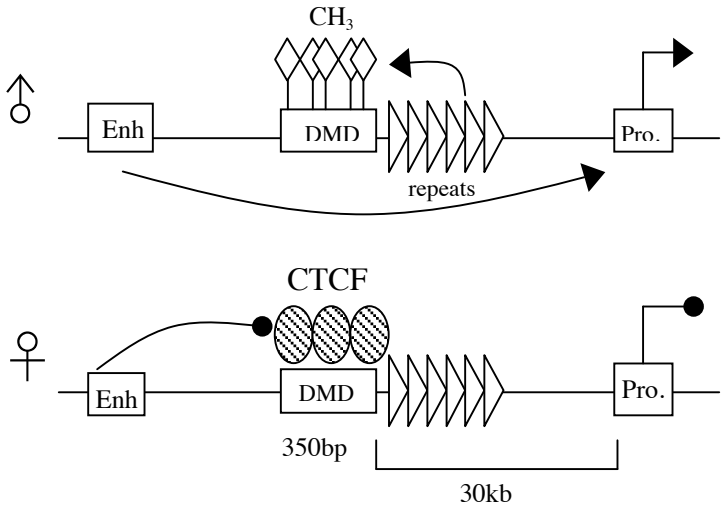


FIGURE I.1: Allele-specific methylation at the DMD governs allele-specific *Rasgrf1* expression in neonatal mouse brain. The paternal DMD is methylated; the maternal DMD is not. CTCF binds to the maternal DMD and blocks endogenous enhancer-promoter interactions, leading to silencing of the maternal allele.

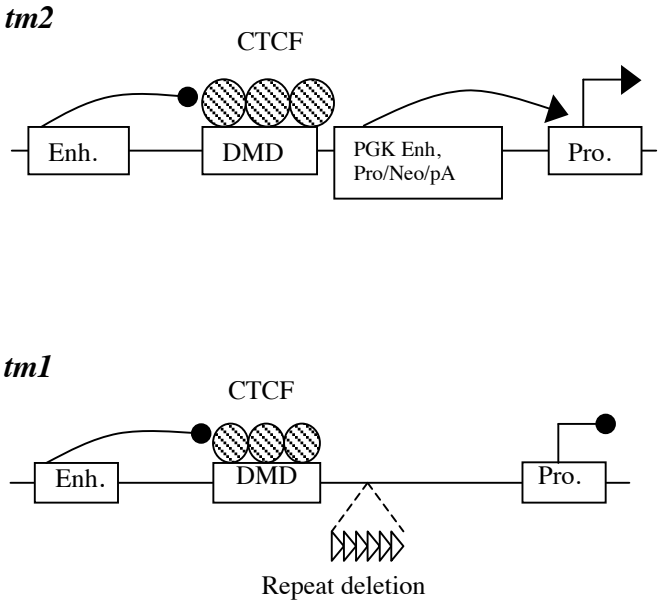


FIGURE I.2: Schematic of *tm1* and *tm2* alleles. The *tm2* allele contains an inserted cassette in place of the repeats and the *tm1* allele contains a repeat deletion.

The sequence elements controlling imprinting at *Rasgrf1* are absent in humans; therefore, *Rasgrf1* is presumably not imprinted. However, *Rasgrf1* expression is imprinted in other murine relatives of mice, including rats.

I.3.2 Expression & Phenotypes

Rasgrf1 is located on mouse chromosome 9 and encodes a 140kD protein. In mouse, *Rasgrf1* is imprinted and expressed preferentially from the paternal allele in neonatal mouse brain (Plass *et al.*, 1996); at weaning, expression at the locus becomes biallelic (Drake *et al.*, 2009), with active maternal allele transcription that persists through adulthood. *Rasgrf1* is predominantly expressed in brain and is absent from most other somatic tissues, with the exception of low levels of imprinted expression in heart, lung, stomach (Plass *et al.*, 1996; Ferrari *et al.*, 1994; Zippel *et al.*, 1997) and pancreas, where the imprinting status is unknown (Arava *et al.*, 1999, Guerrero *et al.*, 1996; Font de Mora *et al.*, 2003). Upstream of *Rasgrf1* are two ncRNAs, *A19* and *AK029869*, that are paternally expressed but have unknown functions (de la Puente *et al.*, 2002; Brideau *et al.*, in prep). A variety of pleiotropic phenotypic effects are associated with loss of *Rasgrf1* expression, including postnatal growth retardation, decreased levels of circulating IGF-1, impaired performance on learning and memory tasks, and electrophysiological differences in neuronal signaling (Itier *et al.*, 1998; Brambilla *et al.*, 1997; Giese *et al.*, 2001; Tonini *et al.*, 2001; Clapcott *et al.*, 2003; Font de Mora *et al.*, 2003; Drake *et al.*, 2009).

I.3.3 Protein function and signaling

RasGRF1 acts as a guanine nucleotide exchange factor (GEF) for the small G-proteins Ras and Rac (Cen *et al.,* 1993; Innocenti *et al.,* 1999; Kiyono *et al.,* 2000) by catalyzing the exchange of inactive, bound GDP for activating GTP. GTPase activating proteins (GAPs) catalyze the opposing reaction, where GTP is exchanged for GDP (Boguski & McCormick, 1993). The RasGRF1 protein contains a catalytic CDC25 domain at the C-terminal of the protein, Dbl- and pleckstrin-homology (DH/PH) domains at the N-terminal, and REM and IQ regions more centrally (**Figure I.3**).

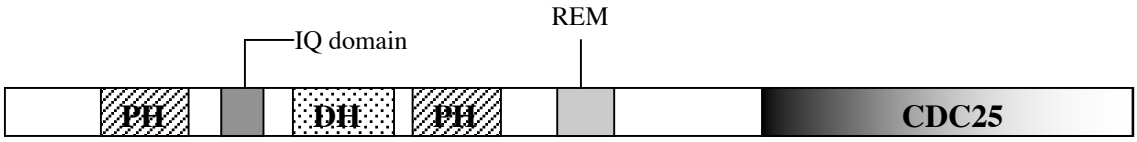


FIGURE I.3: Structure of RasGRF1 protein indicating relative placement of pleckstrin-homology (PH), Dbl-homology (DH), ilimaquinone (IQ), REM, and CDC25 regions.

The CDC25 portion of the protein is responsible for activation and signaling through Ras pathways, and it can act as an exchange factor for H-Ras, K-Ras, N-Ras, and R-Ras (Gotoh *et al.,* 1997; Tian & Feig, 2001). The DH/PH tandem domain is responsible for activation and signaling through Rac pathways (Yang & Mattingly, 2006). Activation of the RasGRF1 protein itself occurs principally in response to calcium influx or serine phosphorylation. Multiple serine residues are phosphorylated in a PKA-dependent manner (Mattingly *et al.,* 1999; Yang *et al.,* 2003), while the REM domain is responsible for autophosphorylation. Calcium signals are sensed through the IQ domain (Farnsworth *et al.,* 1995), though intact PH and coiled-coil domains are necessary for efficient calcium-sensitive regulation

(Buchsbaum *et al.*, 1996). RasGRF1 itself localizes to the plasma membrane as well as the endoplasmic reticulum, where it activates H-Ras in a calcium-sensitive manner (Arozarena *et al.*, 2004).

RasGRF1 responds to several upstream signaling inputs: neurotrophin binding to TrkA, B, and C receptors (Macdonald *et al.*, 1999; Robinson *et al.*, 2005), lysophosphatidic acid (LPA; Mattingly *et al.*, 1999; Zippel *et al.*, 1996 & 2000), heterotrimeric G-protein subunit dissociation (Shou *et al.*, 1995; Kiyono *et al.*, 1999), and muscarinic receptors (Mattingly & Macara, 1996). Downstream signaling effectors are linked to Ras and Rac signaling, and include the Rac/p38 (Baldassa *et al.*, 2007), P13K/Akt (Yang & Mattingly, 2006) and Ras/ERK (Farnsworth *et al.*, 1995; Tian *et al.*, 2004; Tian & Feig, 2006) pathways. RasGRF1 has been shown to directly associate with the NR2B subunit of NMDA-type glutamate receptors (Krapvinsky *et al.*, 2003), which couples NMDA activity to downstream ERK pathways, the GluR1 subunit of the AMPA receptor (Tian & Feig, 2006), microtubules (Forlani *et al.*, 2006), as well as RNA (Lavagni *et al.*, 2009)

I.3.4 *Rasgrf1* and *Rasgrf2*

Rasgrf1 shares a high degree of sequence and peptide similarity with *Rasgrf2*, and non-imprinted gene located on mouse chromosome 13 that codes for a 135kD protein (Fam *et al.*, 1997). Similarities at the peptide level range are between 50% and 88% for each of the protein motifs, with the main distinctive difference between the proteins being an additional 57 amino acids located N-terminal to the CDC25 domain in RasGRF1. Similarly to *Rasgrf1*,

Rasgrf2 expression is enriched in neurons in the central nervous system, though with a different distribution of expression within in the brain (Allen Brain Atlas). Loss of *Rasgrf2* appears to be dispensible for normal growth and maturation in mice, as *Rasgrf2* -/- mice displayed no differences in development, growth, or fertility when compared to wild-type littermates (Fernandez-Medarde *et al.*, 2002). However, both proteins act in concert with the same small G-proteins and associated signaling cascades (Fan *et al.*, 1998), which complicates analysis in the absence of an appropriately-specific method of separating inputs from the two proteins. A commercially-available antibody that can reliably detect *Rasgrf1* in the absence of any cross-reaction with *Rasgrf2* is unavailable (see Appendix); this makes the identification of unique interactors via pull-down assay difficult, as well as complicates any protein-based immunohistochemical imaging.

Previous work in mouse has demonstrated that *Rasgrf1* and *Rasgrf2* mediate opposing forms of synaptic plasticity by influencing different hippocampal electrophysiological processes (Li *et al.*, 2006). In adult hippocampal neurons, RasGRF1 associates with NR2B-containing NMDA receptors and promotes long-term synaptic depression (LTD) by signaling through Rac/p38 pathways. Conversely, RasGRF2 associates with NR2A-containing NMDA receptors and promotes long-term potentiation (LTP) by signaling through Ras/MAPK pathways. Additionally, the degree to which either protein is active in neuronal processes is developmentally-regulated: both RasGRF1 and RasGRF2 become functional in NMDA-induced Ras/Erk signaling in more mature mice (post-natal day 20; Tian *et al.* 2004), a pattern which is seen for AMPA-induced Ras/ERK signaling as well, with activity

commencing around post-natal day 30 (Tian & Feig, 2006). However, both of these latter two studies employed whole-brain protein preparations that may have diluted the effect of *Rasgrf1* activity in a particular structure of importance.

I.3.5 Transient perturbations in *Rasgrf1* Imprinted Expression

We have generated several mutant mice using the *tm1* and *tm2* alleles that perturb *Rasgrf1* imprinting in neonatal mice (Yoon *et al*., 2005). In combination, these engineered alleles produce four patterns of imprinted expression that can be used to assay the effects of disrupting imprinting during a specific developmental period (pre-weaning; **Table I.1**). In the wild-type mouse, *Rasgrf1* is expressed paternally pre-weaning (termed "monoallelic-paternal"); a paternally-inherited repeat-deletion, combined with a wild-type maternal allele produces a mouse with no neonatal *Rasgrf1* expression (+/*tm1*; "null"); a maternally-inherited extra-enhancer allele, combined with a wild-type paternal allele produces a mouse with biallelic neonatal *Rasgrf1* expression (*tm2*/+; "biallelic"); lastly, a materally-inherited extra-enhancer allele, combined with a paternally-inherited repeat-deleted allele produces a mouse with materally-derived monoallelic *Rasgrf1* expression (*tm2*/*tm1*; "monoallelic maternal"). Significantly, biallelic expression at weaning is preserved in each of these genotypes, which recapitulates the endogenous expression pattern for the locus.

TABLE I.1: Alleles generating different *Rasgrf1* imprinted expression patterns in neonatal mice. All genotypes biallelically express *Rasgrf1* as adults.

Allele		Neonatal expression		Nomenclature	Adult expression	
♀	♂	♀	♂		♀	♂
+	+	-	+	Monoallelic paternal, MP (= wt)	+	+
tm2	+	+	+	Biallelic, B	+	+
+	*tm1*	-	-	Null, N	+	+
tm2	*tm1*	+	-	Monoallelic maternal, MM	+	+

These alleles are unique in that they facilitate a study of how traits responsive to imprinted *Rasgrf1* expression are affected not only by a transient loss of *Rasgrf1* expression, but also by expression from the opposite parental allele, as well as overexpression (from the endogenous locus). The *tm2*/*tm1* genotype represents an inversion of imprinting such that expression is still monoallelic, but derived from the opposite parental genome, which facilitates the testing of parental genome-dependent phenotypes, the key untested assumption that the 'conflict hypothesis' uses as a foundation. Additionally, these alleles allow us to ascribe responsibility for certain traits to expression differences that only exist pre-weaning, when *Rasgrf1* is imprinted, as expression still becomes biallelic around weaning. Prior published work has made use of permanently-inactivated null alleles, where loss of *Rasgrf1* expression is maintained through adulthood, thus the phenotypes assayed in adults cannot be ascribed to the activity of the locus during the developmental state in which expression is imprinted.

I.4. Phenotypic consequences of aberrant *Rasgrf1* expression

I.4.1 Learning and Memory

Rasgrf1 is highly and preferentially expressed in neurons, where it is located at synaptic junctions (Zippel *et al.*, 1997; Sturani *et al.*, 1997). This expression pattern led to the hypothesis that *Rasgrf1* played a role in learning and memory processes, and indeed a number of studies have demonstrated the involvement of *Rasgrf1* in synaptic communication, electrophysiological

parameters associated with neuronal signaling, and behaviors assayed in mouse (Brambilla *et al.*, 1997; Tonini *et al.*, 2001; Giese *et al.*, 2001; Tian *et al.*, 2004; Li *et al.*, 2006; Tian & Feig, 2006; Fernandez-Medarde *et al.*, 2007). Additionally, through its association with both neurotrophins and Rac signaling, *Rasgrf1* is involved in neuronal remodeling and the formation of synapses as it contributes to the process of neurite outgrowth and extension (Macdonald *et al.*, 1999; Robinson *et al.*, 2005; Yang & Mattingly, 2006; Baldassa *et al.*, 2007), suggesting that *Rasgrf1* is an important player both in maintaining proper synaptic communication, as well as regulating neuronal remodeling that occurs during development and upon learning.

I.4.1.1 Behavioral Analysis

Studies of the effects of *Rasgrf1* deletion on learning and memory have made use of permanently null *Rasgrf1* alleles, and tested for learning and memory changes in adult animals using well-established behavioral paradigms. The first study published determined that *Rasgrf1* -/- mice display abnormal electrophysiological measurements in the amygdala, as well as impaired performance on a variety of amygdala-dependent tasks, including cued and contextual fear conditioning, and (tone/shock) active and (light/dark) passive avoidance (Brambilla *et al.*, 1997). Performance on hippocampal-dependent spatial-learning tasks (Morris water maze, radial arms maze) was not different from wild-type performance, indicating that Rasgrf1 plays a role in amygdala-dependent memory consolidation, and not short-term memory or 'learning'.

However, a second behavioral study using adult *Rasgrf1*-/- mice produced contradictory results (Giese *et al.*, 2001). Performance on amygdala-dependent tasks (light/dark inhibitory avoidance and tone/shock contextual conditioning) appeared to be normal, with the behavioral effects of *Rasgrf1* deletion manifesting as impairment on hippocampal-dependent tasks (contextual discrimination, social transmission of food preference, hidden-platform Morris water maze). These studies disagree over whether loss of *Rasgrf1* expression primarily affects the amygdala or the hippocampus, but there were variations in behavioral paradigms as well as the Rasgrf1 deletion. However, the conclusion that *Rasgrf1* plays a role in learning and memory is still valid.

I.4.1.2 Cellular Analysis

On a cellular level, *Rasgrf1* deletion appears to affect the processes known as long-term potentiation (LTP) and long-term depression (LTD; Brambilla *et al.*, 1997; Li *et al.*, 2006), both of which are essential for the proper synaptic remodeling that occurs with learning experiences. LTP is thought to be one the cellular processes underlying learning and memory, and it is manifested as the strengthening of a synaptic connection in response to simultaneous stimulation of both the pre- and post-synaptic neurons. Gene transcription and expression in the neuronal nucleus are necessary for the remodeling of synapses that occurs with LTP. Mice with *Rasgrf1* deletions display abnormal amygdalar LTP (Brambilla *et al.*, 1997). However, mice with *Rasgrf1* deletions also display abnormal long-term depression (Li *et al.*, 2006). LTD is an activity-dependent reduction in synaptic strength – distinct from

synaptic weakening – and is also thought to be a significant cellular contributor to synaptic plasticity and learning. Additionally, these mice display difficulties in activating the Ras/MAPK/CREB pathway in response to activation of NMDA and AMPA receptors, which is important for activating gene transcriptional events in response to learning stimuli, a requirement for protein synthesis-dependent neuronal remodeling. Somewhat contradictory is the observation that *Rasgrf1* null mice display an increase in hippocampal neuronal excitability, as well as an increased susceptibility to drug-induced seizures (Tonini *et al.*, 2001). However, hyperexcitability may have effects that are as detrimental to ionic conductances and synaptic transmission as hypoexcitability.

All of the studies assessing the effects of *Rasgrf1* deficiency on neuronal function – at both cellular and behavioral levels – have used a completely null *Rasgrf1* allele and adult animals to draw conclusions. Therefore, while the results of these studies certainly point to a role for *Rasgrf1* in processes involved in learning, memory, and behavior, they do not identify what the function of *imprinted* Rasgrf1 expression early in life is.

I.4.2 *Rasgrf1*, Growth, and Body Weight

Rasgrf1 was the first imprinted gene identified to play a role in postnatal growth (Itier *et al.*, 1998). The "conflict hypothesis" necessarily predicts that imprinted genes will be important for growth, yet prior to *Rasgrf1* being shown to produce a postnatal phenotype, only prenatal growth

had been identified as being affected by imprinted loci (*Igf2* and *Igf2r*, for example; DeChiara *et al*., 1990; Ludwig *et al*., 1996).

Similar to the *Rasgrf1* knockout alleles generated for the study of learning and memory, those used to assess the role of *Rasgrf1* in growth are also permanently inactivating null mutations (Itier *et al.* 1998, Giese *et al.*, 2001, Clapcott *et al.*, 2003, Font de Mora *et al.* 2003). Therefore, while the contribution of *Rasgrf1* expression to growth is clearly demonstrated, the importance of imprinted expression in early life can only be inferred from the data and has not been previously directly demonstrated, nor has the result of *Rasgrf1* overexpression been tested.

Animals null for *Rasgrf1* display a 15-30% reduction in size relative to age-matched wild-type animals, and this difference is apparent shortly after birth (Itier *et al*., 1998; Clapcott *et al*., 2003; Font de Mora *et al*., 2003). Variations in the magnitude of the reduction are produced by differing strain backgrounds and type of mutation, as *Rasgrf1* null alleles used in these studies were generated either via ENU mutagenesis (Clapcott *et al.*, 2003) or by targeted deletion of exons (Giese *et al*., 2001). Significantly, only heterozygous animals inheriting a paternal null allele (+/-) exhibit a reduction in growth (Clapcott *et al*., 2003). Maternal inheritance of a null allele (-/+) produces no phenotype. These patterns suggest that neonatal *Rasgrf1* expression plays a role of paramount importance in the determination of adult body size, and that *Rasgrf1* expression post-weaning is largely irrelevant for this phenotype. If adult expression were important, then one might predict to see an attenuation in size differences in (+/-) animals once maternal allele expression

commenced at weaning; this is not observed. Additionally, in (-/+) animals, one might expect to see a difference in size appear at weaning due to the absence of normal maternal allele expression; this pattern is also not observed. Therefore, one can infer that neonatal *Rasgrf1* expression plays the most significant role in the production of normal body weight; however, from these results it is unclear whether the level of neonatal *Rasgrf1* expression is important, or whether the parent from whom expression is derived is significant.

The link between neuronal *Rasgrf1* expression and growth is likely to be indirect; several candidate downstream effectors have been identified, though no previous work has elucidated a clear connection between *Rasgrf1* and any of them. *Rasgrf1* null mice display decreased levels of circulating IGF-1, and reduced pituitary growth hormone levels (Itier *et al.*, 1998), decreased levels of circulating insulin and IGF1, and increased rates of lipid catabolism (Font de Mora *et al.*, 2003). However, differences in growth appear not to result from differences in metabolism but rather from a change in overall growth regulation processes.

I.4.2.1 The growth hormone/IGF-1 (GH/IGF-1) axis

The growth hormone/IGF-1 (GH/IGF-1) axis is a major regulator of postnatal overall body growth and development, with effects on every major body tissue and organ (Lupu *et al.*, 2001). The contribution of IGF-1 to postnatal growth and development begin immediately after birth, whereas the effects of growth hormone-dependent processes appear shortly after weaning

(Meyer *et al.*, 2004). Because differences in body size observed between *Rasgrf1* null and wild-type mice are apparent before weaning (Drake *et al.*, 2009), IGF-1 and its interactors appear to be likely candidates for involvement in the link between between *Rasgrf1* and growth.

I.4.2.1.1 *Igf1*

IGF-1 (also known as somatomedin c) is a 7.6kD protein that exists in two isoforms that use of two different promoters within the *Igf1* genomic sequence. Additional sequence variants are produced by alternative splicing at the 3′ end of the transcript as well. Isoform 1 is transcribed from the promoter most 5′ of the entire coding region; during splicing, exon 2 is removed. Isoform 2 transcription begins 5′ of exon 2, leading to the omission of exon 1 from the transcript. At the 3′ portion of the transcript, exon 5 is variably included or excluded, producing transcripts with either 4 or 5 exons (Dobrowolny *et al.*, 2005). However, despite the complexities of *Igf1* at the transcript level, both isoforms contain the same insulin-like peptide regions that confer functionality for the mature protein; there are differences in isoform distribution both with age and tissue type. *Igf1* transcription is initiated with the participation of transcription factors including Stat5b (Hosui & Hennighausen, 2008). The signals leading to activation of this pathway are well-studied in relation to the production of isoform 2 in the liver, but are less well-understood in systems leading to isoform 1 transcription in target tissues.

IGF-1 is primarily released from the liver in response to growth hormone signals derived from the pituitary, and isoform 2 predominates in

circulation, where it is complexed with a number of IGF binding proteins (IGFBPs) and the acid-labile subunit (ALS) which act to both prolong the protein's half-life and sequester it, rendering it inactive. From the liver, IGF-1 is secreted and can travel to target tissues, bind to the IGF-1 receptor, and stimulate signal transduction cascades involving such downstream effectors as PI3K and Akt, both of which promote cell division. In addition to liver-derived IGF-1, IGF-1 isoform 1 produced within the target tissues themselves. This locally-produced isoform acts to promote growth in an autocrine manner, though its contributions to overall growth and development are less significant than those of the circulating form. However, there is emerging evidence indicating that locally-derived IGF1 may play a more important role in growth than initially thought, as increased levels of isoform 1 can rescue some of the phenotype produced by the loss of liver-derived IGF1 (Yakar *et al.*, 1999).

I.4.2.2 Regulation of the GH/IGF-1 axis

The GH/IGF-1 axis is neuronally-regulated and properly maintained through the existence of feedback loops. Though IGF-1 is ultimately released from the liver and can serve as an indicator for proper axis function, the upstream components of the axis originate within the ventromedial hypothalamus. The hypothalamus is a structure located at the base of the brain that integrates signals and plays a role in a number of homeostatic processes including regulation of appetite and feeding behavior, hormonal signaling, body temperature, and circadian rhythms. Growth hormone releasing hormone (GHRH) is produced in the arcuate nucleus of the

ventromedial hypothalamus and travels to the pituitary through the hypophyseal portal – a structure comprised of both neurons and blood vessels. In the pituitary, GHRH signals via its receptor (GHRH-R) to promote the synthesis and release of growth hormone (GH), which is secreted and signals via its receptor (GH-R) in the liver to promote IGF-1 secretion (**Figure I.4**). From there, IGF-1 is released into circulation and reaches target tissues (bone, muscle, organs) where it signals via its receptor (IGF1-R) and stimulates a number of processes including bone growth and elongation, muscle cell development, and organogenesis. Feedback loops maintain the proper level of IGF-1 in circulation by affecting the level of GHRH in the hypothalamus, as well as the level of somatostatin, a peptide which acts to inhibit signaling through the axis and reduce the amount of IGF-1 in circulation.

Transcription factors known to play a role in regulating the major players in this axis are beginning to be understood, though the complexity of the axis necessarily creates difficulties in identifying the network of interactions.

As mentioned previously, the GH/IGF-1 axis plays a major role in postnatal growth, with IGF-1 levels increasing from birth onward, though the relative amounts of each isoform vary with age, with isoform 2 becoming more abundant with maturation. IGF-1 and IGF1-1 receptor knockout mice display growth retardation immediately upon birth, and complete knockouts exhibit a host of other phenotypes including respiratory distress and a high percentage of postnatal lethality (Liu *et al.*, 1993, 1998). Heterozygotes display an intermediate reduction in size. GH and GH-receptor knockout mice are

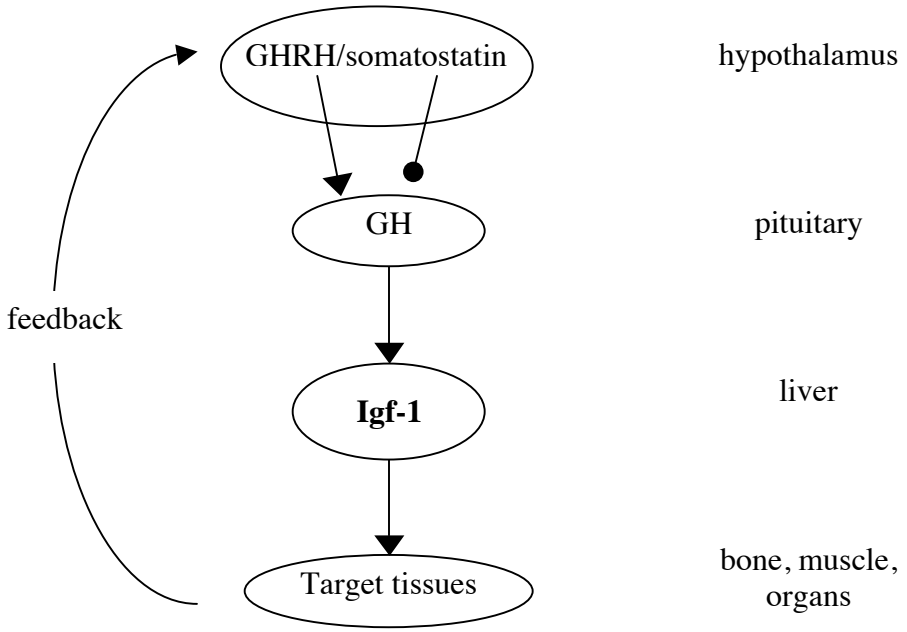


FIGURE I.4: Schematic of the growth hormone/IGF-1 axis. Structures are indicated on the right, and axis components are circled.

normally-sized at birth but display significant size deficits commencing around weaning (Zhou *et al.*, 1997; Meyer *et al.*, 2004). GHRH knock out mice are reduced in size shortly after birth, have lower levels of IGF-1 in circulation, and decreased amounts of pituitary growth hormone (Alba & Salvatori, 2004). Overexpression of pathway components leads to modest increases in size, though not of the magnitude one might expect, perhaps due to the presence of well-developed feedback loops.

I.5 Present studies

I intend in this dissertation to present work furthering the understanding of the role imprinted *Rasgrf1* expression plays in the presentation of growth and learning phenotypes in mouse. These two principal phenotypes will be elaborated upon, with identification of pathways linking each phenotype to imprinted *Rasgrf1* expression. I make use of the transient imprinting mutants to help establish whether each phenotype is dependent upon proper imprinting in neonatal mice, as the importance of genomic imprinting in producing a number of phenotypes is just beginning to be understood.

II. IMPRINT SWITCH MUTATIONS AT *RASGRF1* SUPPORT CONFLICT HYPOTHESIS OF IMPRINTING AND DEFINE A GROWTH CONTROL MECHANISM UPSTREAM OF IGF1[1]

II.1 Abstract

Rasgrf1 is imprinted and expressed preferentially from the paternal allele in neonatal mouse brain. At weaning, expression becomes biallelic. Using a mouse model, we assayed the effects of perturbing imprinted *Rasgrf1* expression in mice with the following imprinted expression patterns: monoallelic-paternal (wild-type), monoallelic maternal (maternal-only), biallelic (both alleles transcribed), and null (neither allele transcribed). All genotypes exhibit biallelic expression around weaning. Consequences of this transient imprinting perturbation are manifested as overall size differences that correspond to the amount of neonatal *Rasgrf1* and are persistent, extending into adulthood. Biallelic mice are the largest and overexpress *Rasgrf1* relative to wild-type mice, null mice are the smallest and underexpress *Rasgrf1* as neonates, and the two monoallelically-expressing genotypes are intermediate and indistinguishable from one another, both in size and in *Rasgrf1* expression level. Importantly, these data support one of the key underlying assumptions of the "conflict hypothesis" which describes the evolution of genomic imprinting in mammals, and supposes that equivalent amounts of imprinted gene expression produce equivalent phenotypes, regardless of which parental allele is transcribed. Concordant with the

[1] Drake NM, Park YJ, Shirali AS, Cleland TA and Soloway PD (2009) Imprint switch mutations at Rasgrf1 support conflict hypothesis of imprinting and define a growth control mechanism upstream of IGF1. *Mammalian Genome*, epub ahead of print, 10 June 2009; doi:10.1007/s00335-009-9192-7

difference in overall body size, we identify differences in IGF-1 levels, both in serum protein and as liver transcript, and identify additional differential expression of components upstream of IGF-1 release in the GH/IGF-1 axis. These data suggest that imprinted *Rasgrf1* expression affects GH/IGF-1 axis function, and that the consequences of *Rasgrf1* inputs to this axis persist beyond the time period when expression is restricted via epigenetic mechanisms, suggesting that proper neonatal *Rasgrf1* expression levels are critical for development.

II.2 Introduction

Imprinted genes are expressed preferentially from one of two parental alleles in a predetermined manner. Epigenetic mechanisms distinguish the two alleles from one another and control imprinted expression, which can be tissue-specific or restricted to certain developmental stages. Imprinted genes have been identified in a variety of eutherian and meta-eutherian mammals (Wiedman *et al.*,2004; Suzuki *et al.*,2005; Edwards *et al.*, 2007), suggesting that imprinted loci have somehow escaped the protection diploid expression provides against recessive mutations, though the possibility exists that imprinting might evolve at loci where deleterious mutations are the least likely to be recessive (Ubeda & Wilkins, 2008).

A widely-accepted theory explaining the emergence of genomic imprinting in mammals is the "conflict" or "kinship" hypothesis (Moore & Haig, 1991). It postulates that conflict exists between parental genomes regarding the transfer of maternal resources to offspring, with paternal reproduction benefiting from abundant transfer and maternal reproduction

benefiting from parsimonious transfer. At loci that control this transfer, the gradual tug-of-war between the genomes over optimum expression levels ultimately results in imprinted monoallelic expression. Maternally-expressed imprinted genes are predicted to act as growth inhibitors in offspring, and paternally-expressed imprinted genes are predicted to act as growth promoters.

Rasgrf1 is an imprinted gene that is expressed preferentially from the paternal allele in neonatal mouse brain, with maternal allele expression identified in adults (Plass *et al.*, 1996); expression is high in the central nervous system, though low levels of expression have been identified in other tissues (Plass *et al.*, 1996). RasGRF1 is a 140kDa protein that acts as a guanine-nucleotide exchange factor and activates the small g-proteins Ras and Rac (Innocenti *et al.*, 1999; Yang & Mattingly, 2006) in a calcium-dependent manner (Farnsworth *et al.*, 1995). As its expression pattern might suggest, *Rasgrf1* plays a role in learning and memory (Brambilla *et al.*, 1997; Giese *et al.*, 2001) and associates with NMDA and AMPA receptors in neurons (Krapivinsky *et al.*, 2003; Tian *et al.*, 2004; Tian & Feig, 2006).

As predicted by the conflict hypothesis, *Rasgrf1* is also known to play a role in postnatal growth. Mice completely lacking *Rasgrf1* expression display a moderate 15-30% reduction in weight that varies depending on the mouse strain, type of mutation introduced, sex and age assayed (Itier *et al.*, 1998; Giese *et al.*, 2001; Clapcott *et al.*, 2003).

Studies completed previously have all used permanent *Rasgrf1* null alleles; thus, none have considered the consequences of transiently perturbing *Rasgrf1* expression during the neonatal period, which is when *Rasgrf1* expression is imprinted. Using two mouse models previously described (Yoon *et al.*, 2002; Yoon *et al.*, 2005), in which *Rasgrf1* imprinted expression is disrupted, we have assayed the importance of proper neonatal imprinted expression. Additionally, combining these models produces an imprinting switch, in which expression is maternally-derived with corresponding paternal allele silencing. This unique feature allowed us to test the equivalence of expression derived from either parental allele; more specifically, we could ask whether equivalent phenotypes arise when equivalent amounts of expression are maternally or paternally contributed, which is a critical assumption that the conflict hypothesis rests on (Wilkins & Haig, 2003). The hypothesis assumes that genomic imprinting in mammals emerged solely as a means of controlling the levels of total imprinted gene expression, and that there are no qualitative differences between expression derived from the two parental alleles. Our series of mutant alleles at the *Rasgrf1* imprinted locus afforded us the opportunity to test whether this assumption is valid, in addition to asking whether neonatal misimprinting has phenotypic consequences.

Two mutated *Rasgrf1* alleles – termed *tm1* and *tm2* – transiently impart three additional patterns of imprinted expression in neonates (Yoon *et al.*, 2002; Yoon *et al.*, 2005). Imprinted expression at the *Rasgrf1* locus is controlled by methylation at a differentially methylated domain (DMD) 30kb upstream of the gene's promoter; the DMD is flanked 3′ by a series of repeats that direct

the placement of methyl groups on the paternal allele DMD. When these repeats are deleted – as they are in the *tm1* allele – the DMD fails to acquire methylation and expression is silenced. An unmethylated DMD is a target for CTCF binding, which acts as an enhancer-blocker and inhibits communication between the gene's endogenous enhancer and its promoter. When an extra enhancer is inserted in place of the repeats – as is the case with the *tm2* allele – expression is present even in the absence of DMD methylation because the extra enhancer bypasses the need for endogenous enhancer-promoter communication. Inheriting these two alleles in various combinations produces four different patterns of imprinted neonatal *Rasgrf1* expression: wild-type mice (+/+) exhibit monoallelic-paternal (MP) expression, biallelic mice (B, *tm2*/+) express *Rasgrf1* from both alleles, null mice (N, +/*tm1*) do not express *Rasgrf1* from either allele, and monoallelic-maternal mice (MM, *tm2*/*tm1*) express *Rasgrf1* from the maternal allele. The MM mice represent an inversion of imprinting because *Rasgrf1* expression is still monoallelic, but maternally-derived. Importantly, biallelic expression of *Rasgrf1* is maintained in adult animals from each of these genotypes, mimicking the wild-type relaxation of imprinting at the locus.

We demonstrate here that transient perturbations in *Rasgrf1* imprinted expression significantly affect overall growth, and that the phenotypic effect of imprinting mutations persists into adulthood, beyond the period during which expression is epigenetically restricted. Biallelic animals are the largest, null animals are the smallest, and monoallelic-paternal and -maternal animals are intermediate between the two and indistinguishable from one another. Differences in adult size are sensitive to the overall level of neonatal *Rasgrf1*

expression, not the allele from which expression is derived, which validates an important assumption of the "conflict" or "kinship" hypothesis. We also demonstrate that *Rasgrf1* produces this effect through involvement of the growth hormone/IGF-1 axis, as differences in IGF-1 serum protein and liver transcript levels exist, as well as differential expression of genes upstream of IGF-1 release that are critical for proper functioning of this neuroendocrine axis.

II.3 Materials & Methods

II.3.1 Breeding Scheme

Mice were generated as described previously (Yoon *et al.*, 2002; Yoon *et al.*, 2005), with *tm1* and *tm2* alleles maintained on a C57/Bl6 background. Mice used for initial weight measurements are littermate controls generated by crossing *tm2*/+ females with +/*tm1* males to facilitate maternal transmission of the *tm2* allele and paternal transmission of the *tm1* allele and produce all four genotypes in a given litter. Weaning occurred at day 21. Mice used for subsequent gene expression assays, body composition analyses, and IGF-1 measurements were generated by crossing homozygous mice: *tm2*/*tm2* females were crossed with *tm1*/*tm1* males and +/+ males to generate *tm2*/*tm1* and *tm2*/+ animals, respectively; +/+ females were crossed with *tm1*/*tm1* and +/+ males generated +/*tm1* and +/+ animals, respectively.

II.3.2 Animal measurements

Weights were measured weekly. Length measurements were taken at the same ages using calipers, and included only the body length of the mouse.

Organ weights (liver, spleen, kidney, thymus) were measured immediately post-dissection and normalized to pre-dissection body weight. Body composition was measured via NMR and performed by the University of Cincinnati Mouse Metabolic Phenotypic Center. Meal sizes were assayed using singly-housed P63 adult animals by weighing the amount of food both before and after a 3-day period. Animals were housed in wire-bottom cages so that food particles could be collected from the bottoms and included in the post-trial measurements.

II.3.3 *Rasgrf1* Imprinted Expression

RNA was isolated from the brains of mice between embryonic day 13.5 (e13.5) and adulthood at postnatal day 42 (P42). Mice arose from crosses between 129S4Jae mothers and PWK fathers. PWK has a polymorphism in the 3' untranslated region of the mRNA containing a *HhaI* site that 129S4Jae lacks, so after RT-PCR, PCR products (F: CTTGGT GTTCATCGAGGAGG; R: ATATTCTCGGGGAAGCACAC) can be digested with *HhaI*, and digestion products will reveal which allele is expressed. PCR products generated after cDNA amplification were run on a gel without *HhaI* digestion or after cutting with *HhaI*. Just prior to digestion, the PCR products were mixed with a control DNA fragment (MetFR).

II.3.4 RNA Quantification

For *Rasgrf1* transcript quantification, brains were collected from P11 neonates. RNA was extracted, reverse-transcribed, and quantified in duplicate using ABI Taqman© probes specific for *Rasgrf1* and 18S rRNA, to which *Rasgrf1* expression levels were normalized. *Igf-1* liver transcript was

quantified in triplicate at P21, using RNA extracted from whole liver. An ABI Taqman© probe recognizing both transcript isoforms of *Igf-1* was used, again normalized to 18S rRNA.

Quantification of *Sst*, *Gh*, *Ghrh*, *IgfBp3*, and *Prl-R* were performed using a Sybr-Green based real-time assay. Transcript levels were normalized to *Rpl32*, a ribosomal subunit. Prior to quantification, cDNAs and RNAs were verified to be free from genomic DNA contamination and PCR products were digested with appropriate restriction enzymes to verify identity. Primer pairs: *Gh*: (F) TCC TCA GCA GGA TTT TCA CC (R) GCA GCC CAT AGT TTT TGA GC; *Ghrh*: (F) TGT GGA CAG AGG ACA AGC AG (R) ACA GAG GAC GGA AAA GGT CA; *Igf-1* Isoform 1: (F) CCT GCG CAA TGG AAT AAA GT (R) ATT GAG TTG GAA GGC TGC TG; *IgfBP3*: (F) CGC AGA GAA ATG GAG GAC AC (R) TTG TTG GCA GTC TTT TGT GC; *Prl-R*: (F) ATC ATT GTG GCC GTT CTC TC (R) CCA GCA AGT CCT CAC AGT CA; Rpl32: (F) CAT GCA CAC AAG CCA TCT ACT CA (R) TGC TCA CAA TGT GTC CTC TAA GAA C; *Sst*: (F) GAG GCA AGG AAG ATC CTG TC (R) ACT TGG CCA GTT CCT GTT TC.

II.3.5 IGF-1 Protein Quantification

Serum IGF-1 protein was quantified by a double-labeled radioimmunoassay at Vanderbilt University's Hormone Assay Core, a Mouse Metabolic Phenotyping Center. Blood was extracted from P21 animals, clotted at room temperature for 15minutes, and spun @ 3000g for 15min @4C, after which serum was collected.

II.3.6 Statistics

Weight data were analyzed using a repeated measures analysis of variance (MANOVA). Expression data were analyzed using the student's *t*-test or the nonparametric Mann-Whitney U-test, and p-values were Bonferroni-corrected to account for multiple comparisons.

II.4 Results

II.4.1 *Rasgrf1* Imprinted Expression

We first characterized the temporal component of imprinted *Rasgrf1* expression, as biallelic expression in adult brain tissues was previously identified (Plass *et al.*, 1996). *Rasgrf1* imprinting in brain is relaxed during development and expression becomes biallelic around the time of weaning (**Figure II.1**). The transition from strongly and predominantly paternal allele expression to biallelic expression can be seen over time.

In contrast to previous work in which expression was only identified in postnatal tissues (Ferrari *et al.*, 1994), we detected transcript prenatally, as early as embryonic day 13.5. Next, we sought to quantify *Rasgrf1* levels in neonatal brains from animals of each of the four imprinted expression patterns (n=3 animals/genotype) using standard qPCR with an ABI Taqman© probe specific for *Rasgrf1* (**Figure II.2**). Expression levels are not significantly different between the two monoallelic genotypes in neonatal brain (p=0.30), but are significantly increased in biallelics (p=0.029) and significantly decreased in nulls (p=0.0024) relative to wild-type MP mice. These data demonstrate the equivalence of *Rasgrf1* expression level in the MM and MP

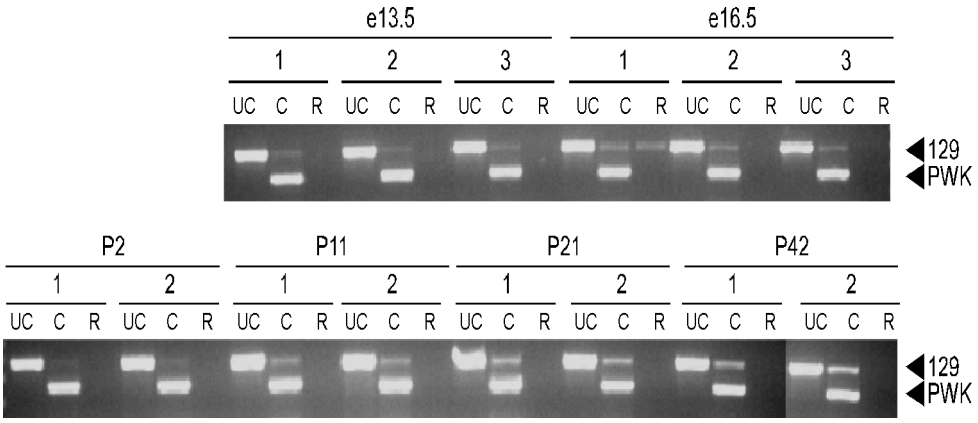


FIGURE II.1: Imprinting at *Rasgrf1* is relaxed during development. RNA was isolated from either two or three brains of mice between embryonic day 13.5 (e13.5) and adulthood at postnatal day 42 (P42). Mice arose from crosses between 129S4Jae mothers and PWK fathers. cDNA (UC and C) or RNA (R) was amplified by PCR. In no cases did RNA support amplification demonstrating the RNA we isolated had no genomic DNA contamination. PCR products made after cDNA amplification were run on a gel without *HhaI* digestion (UC) or after cutting with *HhaI* (C). The PWK PCR product has a *HhaI* site lacking in the 129S4Jae product. Therefore, the quickly-migrating band labeled PWK in blue are diagnostic for paternal allele expression while the slowly migrating species labeled 129 in red is diagnostic for maternal allele expression. The transition from strongly and predominantly paternal allele expression to biallelic expression can be seen over time.

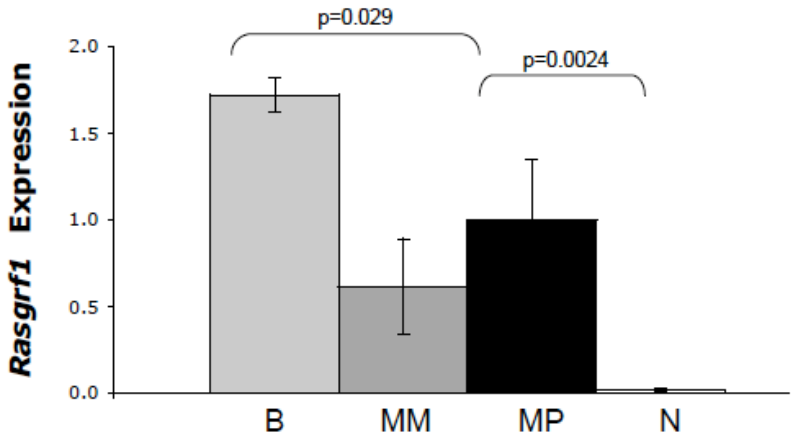


FIGURE II.2: *Rasgrf1* expression in imprinting mutants. Real-time quantification of *Rasgrf1* expression in neonatal whole brain, plotted relative to wild-type (MP) animals. 3 animals/genotype were assayed. P-values (*t*-test) are indicated. Error bars represent standard deviations.

genotypes, as well as the relative over- and under-expression of the B and N genotypes, respectively; they also identify the pre-weaning period as the time during which *Rasgrf1* expression is imprinted.

II.4.2 Adult Body Size Corresponds to Neonatal Rasgrf1 Expression Pattern

We observed a range of size differences among weaned mice of both sexes that appeared to be correlated with transient perturbations in *Rasgrf1* imprinted expression. To reliably assess these differences in size, mice were weighed weekly, between postnatal day 8 and 63, and generated using a breeding scheme that produced all four genotypes in a single litter. A difference in weight was observed that is correlated with neonatal *Rasgrf1* expression level (**Figure II.3**), with relationships among the genotypes mirroring those identified in transcript levels. For both sexes, trends are the same: nulls are significantly lighter than the other genotypes from day 16 onward ($P<0.0001$, MANOVA), biallelics are significantly heavier than the other genotypes from day 16 onward ($P<0.0001$, MANOVA), and wild-type MP mice are intermediate in size between the biallelics and nulls.

Furthermore, differences in weight are proportional to differences in length (**Supplementary Figure II.S1 a, b**), with biallelic mice being both heavier and longer, and null mice being both lighter and shorter, suggesting that we are seeing an overall growth effect and not the effect of metabolic processes affecting weight gain alone. Further support for this conclusion is drawn from the results of a body composition analysis (**Supplementary Figure II.S2**) indicating that differences in body composition, as reported by body fat percentage at P63, do not reflect differences in weight. The only

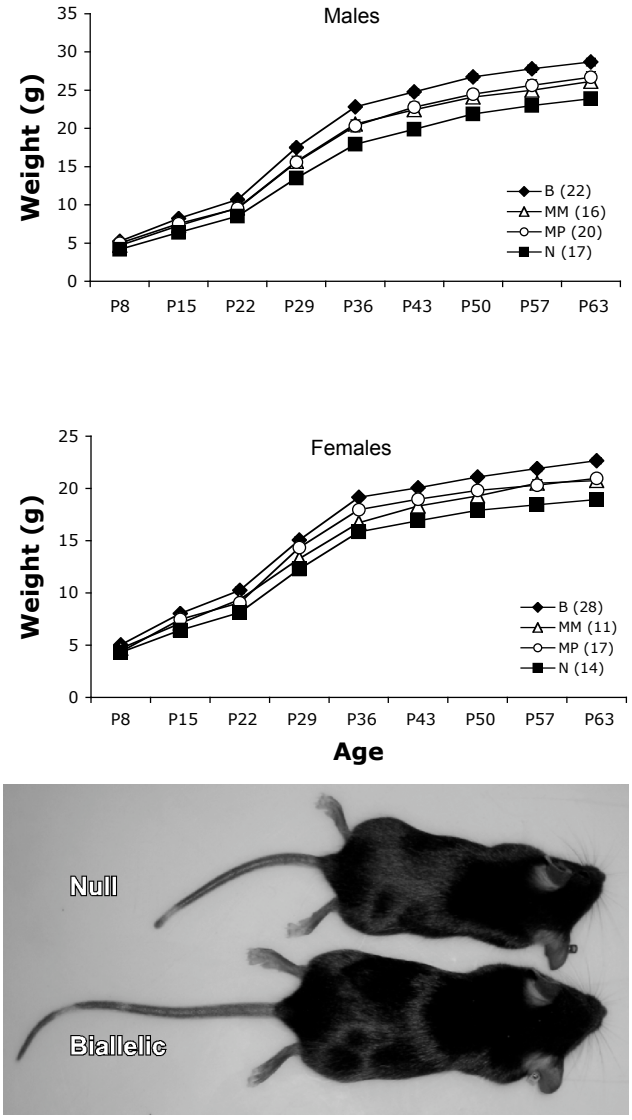
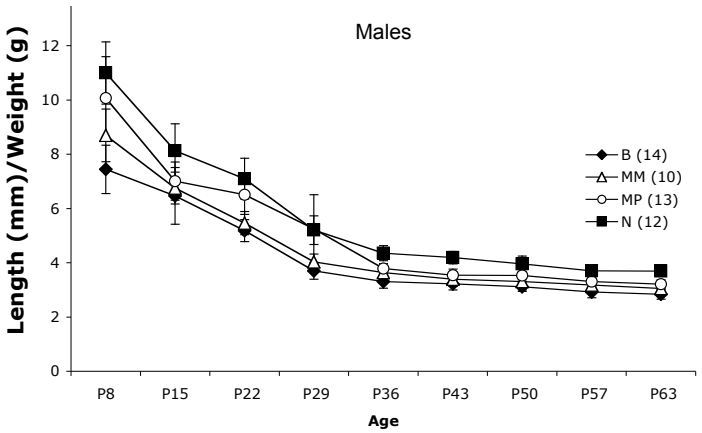
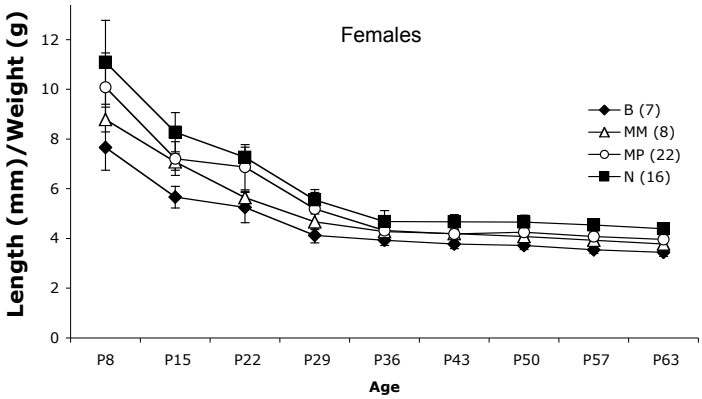


FIGURE II.3: *Rasgrf1* expression level, not parent-of-origin, controls body weight. Male (top) and female (middle) body weights were measured once a week, with the number of animals indicated. Error bars represent standard deviations. In comparison with the MP controls across the full time interval, null mice were significantly lighter ($P<6x10^{-6}$), Bi mice were significantly heavier ($P<2x10^{-4}$), and MM were indistinguishable from controls. Differences between the null and biallelically-expressing mice were highly significant ($P<8x10^{-17}$). Representative female mice at P50 (bottom) from the null and biallelic cohorts.

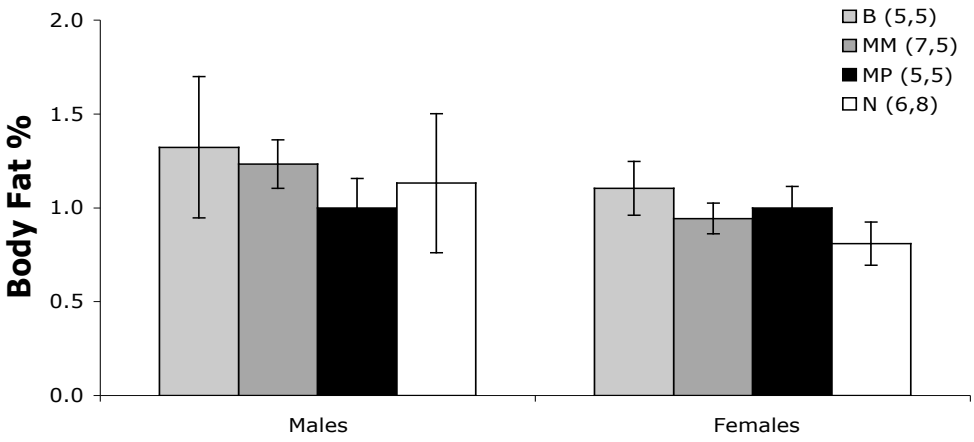
A



B



SUPPLEMENTARY FIGURE II.S1: Differences in weight and length are proportional. The ratio of length(mm) to weight(g) is plotted for both males (a) and females (b) from P8-P63, with the number of animals indicated. Error bars represent standard deviations. Mice were weighed and measured once a week. From P36-P63, there are no differences in the relative proportions of weight and length among genotypes in female mice; in males, only the nulls and wild-type mice are different ($p=0.029$) during this interval. P-values were generated using a repeated measures MANOVA. P-values generated across the entire interval indicate that pre-weaning proportions vary in both sexes, but with the exception of wild-type and null males, adult proportions are comparable.

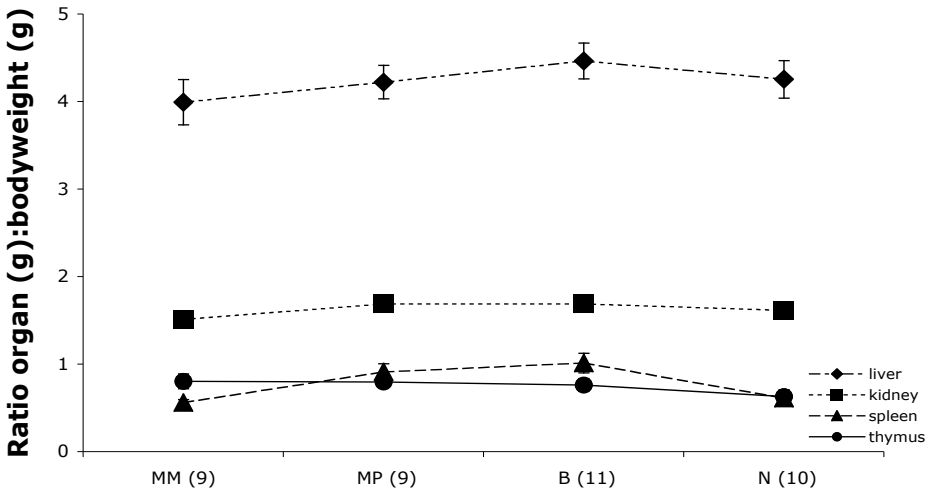


SUPPLEMENTARY FIGURE II.S2: Body Fat% is not reflective of differences in size at P63. Body composition was assayed in P63 mice from each genotype by NMR. Body Fat% was plotted relative to wild-type %, with the number of animals (male, female) indicated. With the exception of female biallelic and null animals (p=0.0018), there were no significant differences in body fat % detected among genotypes of either sex at P63, when weights were significantly different among genotypes of both sexes. Error bars represent standard deviations.

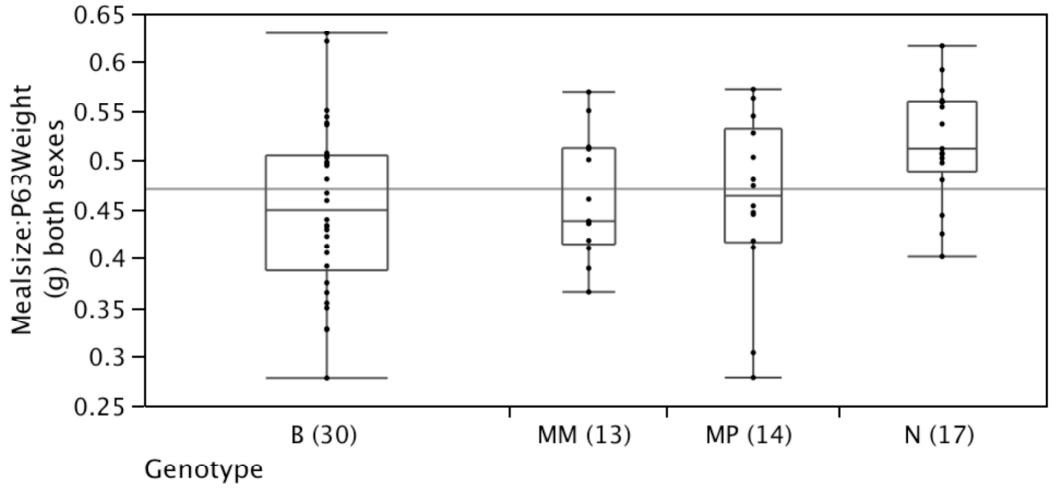
difference identified in body fat % was between female biallelic and null mice (p=0.0018). Additionally, the ratios of various organ weights to total body weight are similar among the four genotypes at P21 (**Supplementary Figure II.S3**).

An assessment of P63 adult meal size revealed that differences in adult body size were not proportional to the amount of food eaten by different genotypes (**Supplementary Figure II.S4**). When food intake is normalized to body weight, null mice are eating more per gram of body weight than are biallelic mice (p=0.0342), suggesting that the reduction in size is not due to a failure to eat.

Overall, these results indicate that differences in adult body size are responsive to the level of neonatal *Rasgrf1* expression, which highlights the importance of proper imprinted expression early in development. Among genotypes, adult body size corresponds to the amount of *Rasgrf1* in neonatal brain, with the larger, biallelic mice overexpressing *Rasgrf1* and the smaller, null mice underexpressing *Rasgrf1*. The monoallelically-expressing genotypes are indistinguishable from one another both in transcript level and body size, and are intermediate between the two extremes, indicating that equivalent levels of expression do produce equivalent phenotypes, as the conflict hypothesis assumes. Of additional importance is the observation that differences in weight persist beyond weaning, when expression from both *Rasgrf1* alleles commences, suggesting that pre-weaning imprinted expression functions in the setting of parameters affecting growth through adulthood by modulating overall *Rasgrf1* expression level.



SUPPLEMENTARY FIGURE II.S3: Organ weights at P21 do not reflect differences in size. Organs from P21 males of all genotypes were dissected, and wet weights were plotted relative to total body weight (numbers of animals per genotype are indicated). There are no significant differences in the ratio of liver:body weight (*t*-test). The log(kidney:bodyweight) ratios (*t*-test) indicated no significant differences among genotypes as well, as did nonparametric analysis (Mann-Whitney U-test) of thymus:body weight ratios. Oddly, the log(spleen:bodyweight) data indicated a significant difference between MM and MP animals ($p=0.0102$), as well as MM and B animals ($p=0.0192$), though this particular difference among genotypes is not seen in any other analysis.



SUPPLEMENTARY FIGURE II.S4: Differences in meal size do not reflect weight differences. Meal sizes were measured over a 3-day period in P63 animals that had participated in the original weight study. Intake for both sexes, normalized to P63 body weight measured are shown. Significant differences were seen by *t*-testing between mice with null expression (N; +/tm1;) and mice with biallelic expression (Bi; *tm2*/+; p=0.0342). Numbers of animals included are indicated.

II.4.3 IGF-1 is decreased in transient *Rasgrf1* nulls, at transcript and protein level

Given the proportional effect of *Rasgrf1* expression on overall growth, we reasoned that a master growth-regulating axis might be affected by *Rasgrf1*. We investigated whether the growth hormone/IGF-1 axis (GH/IGF-1 axis), which is known to play a significant role in regulating post-natal growth (Lupu *et al.*, 2001), might be a target. Circulating growth hormone levels are difficult to capture accurately due to the pulsatile nature of GH secretion, so circulating IGF-1 protein is generally used as an indication for how the system is functioning.

IGF-1 levels were evaluated both as protein and transcript. In serum, IGF-1 protein was measured by radioimmunoassay at postnatal day 21 in males (**Figure II.4a**). P21 animals produce data from around the time that growth becomes GH-dependent (Meyer *et al.*, 2004) and display significant weight differences in both sexes. P21 data indicate a clear deficiency in the amount of IGF-1 circulating protein in the null mice, relative to the biallelic mice ($p=0.0012$). There is no statistically significant overabundance of IGF-1 protein in biallelic animals relative to the intermediate genotypes. However, the clear difference in IGF-1 levels between the largest and smallest animals indicates that IGF-1 is critical to the overall growth phenotype produced by *Rasgrf1* imprinting perturbation.

Decreased IGF-1 serum levels in the null mice appear to be due to a decrease in mRNA accumulation in liver. We quantified *Igf-1* expression in the livers of P21 male animals, using a Taqman© probe that also recognized

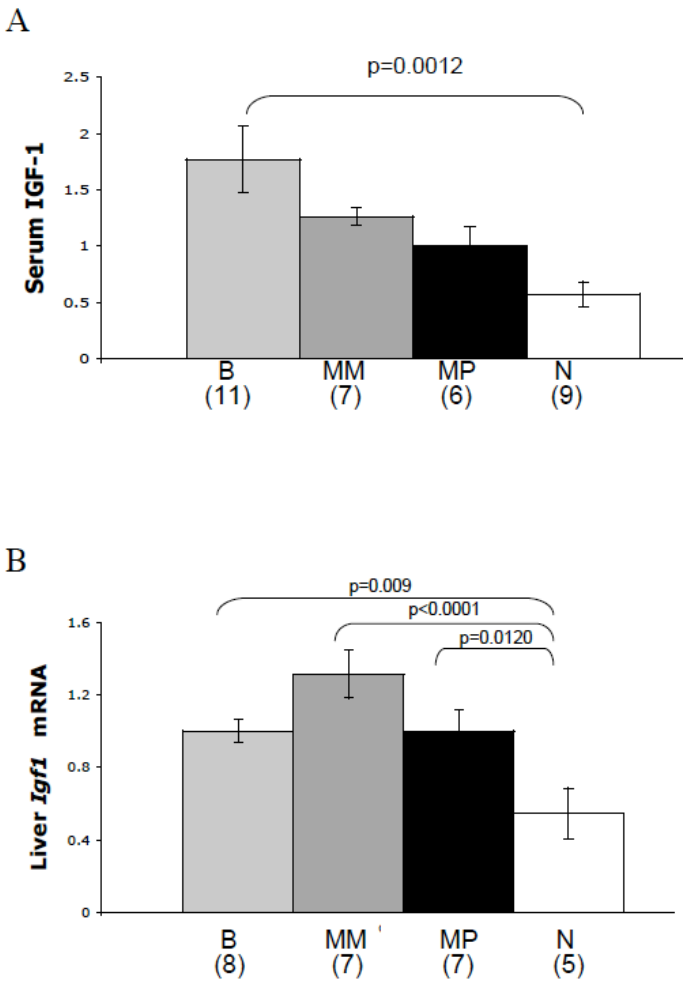


FIGURE II.4: IGF-1 levels are reduced in transient nulls at P21. *Rasgrf1* expression differences are accompanied by differences in IGF-1 levels. (A) P21 IGF-1 serum levels are reduced in transient nulls. Serum levels were quantified by radioimmunoassay in male mice from each genotype, with the number of animals indicated, and plotted relative to MP (wild-type) protein levels. Circulating IGF-1 protein is reduced in transient nulls relative to biallelics (p=0.0012). Error bars represent standard errors. (B) P21 *Igf-1* transcript levels are reduced in transient nulls. Liver *Igf-1* transcript was quantified in male mice from each genotype using a Taqman© probe specific for IGF-1 and normalized to 18S rRNA, with the number of animals indicated, and plotted relative to MP transcript levels. *Igf-1* transcript is reduced in null animals relative to biallelics (p=0.009), monoallelic-maternals (p<0.0001), and monoallelic-paternals (p=0.0120). Error bars represent standard errors.

both IGF-1 isoforms (**Figure II.4b**). Taqman©-based data indicate the same trend present in serum: nulls underexpress *Igf-1* in relation to the other three genotypes (N/B p= 0.009; N/MM p<0.0001; N/MP p=0.012) yet we cannot identify overexpression of *Igf-1* in biallelic mice.

II.4.4 Rasgrf1 affects hypothalamic inputs to the GH/IGF-1 axis

Since IGF-1 levels are affected by differences in neonatal *Rasgrf1* imprinted expression (N<<B, at least, and the difference is in the predicted direction), we raised the question of how *Rasgrf1* expression in the brain might affect IGF-1 transcription and release from the liver. One possibility is that *Rasgrf1* levels may influence the activity of downstream transcription factors that are upstream of IGF-1 release. Given that *Rasgrf1* is expressed preferentially in the central nervous system, and not in the liver, we began an investigation into whether various neural components of the GH/IGF-1 axis are differentially expressed in our mutant mice, perhaps as a consequence of *Rasgrf1* influence on transcription factor activity. If a particular transcription factor were a downstream target of *Rasgrf1*, then differences in the transcripts dependent on that factor might indicate its identity as a target. Reasoning that the differences in *Rasgrf1* and IGF-1 levels are most robust in our two "extreme" genotypes, we limited this analysis to null and biallelic male animals, and plotted transcript quantities relative to nulls.

Transcription factors chosen as targets were CREB, Ikaros, Pit-1, and Stat5 (**Figure II.5**). Transcripts sensitive to CREB activity include somatostatin (*Sst*; reviewed in Montminy *et al.*, 1996) and growth hormone releasing hormone (*Ghrh*; Mutsuga *et al.*, 2001); transcripts sensitive to Ikaros activity

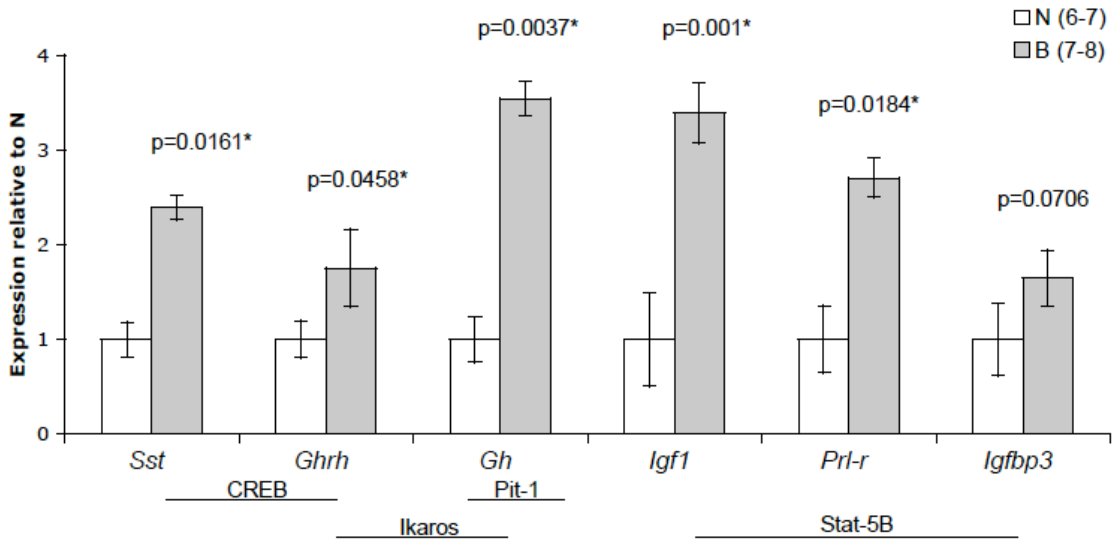


FIGURE II.5: Differential expression of GH/IGF-1 axis components in P21 biallelic and null males. Differential expression of GH/IGF-1 axis components in P21 biallelic and null males. Sybr-Green based quantification of genes involved in GH/IGF-1 axis function, normalized to Rpl32 and plotted relative to expression level in nulls. Numbers of animals are indicated; error bars represent standard errors. Transcription factors responsible for each gene are indicated below. Overexpression in biallelics is seen for the following transcripts: Sst (p=0.0161), GHRH (p=0.0458), GH (p=0.0037), Prl-r (p=0.0184), and IGF-1 (p=0.001), which was included as a control. No significant difference was observed in IGFBP3 level.

include *Ghrh* (Ezzat *et al.*, 2006) and growth hormone (*Gh*; Ezzat *et al.*, 2005); Pit-1 activity is required for *Gh* transcription as well as other pituitary-derived hormones, prolactin and TSHß
(Scully *et al.*, 2000); and Stat5b-dependent transcription occurs in the liver, at the *Igf1*, *Prl-R*, and *Igfbp3* (reviewed in Hosui & Henninghausen, 2008). Significant differences were identified between the biallelic and null animals for somatostatin (hypothalamus; p=0.0161), growth hormone releasing hormone (hypothalamus; p=0.0458), growth hormone (pituitary; p=0.0037), Igf-1 (liver; p=0.001), and prolactin receptor (liver; p=0.0184). IGF1BP3 (liver) did not demonstrate a significant difference.

The most upstream of these components in the GH/IGF-1 axis is growth hormone releasing hormone, which is produced in the hypothalamus and signals via its receptors in the pituitary to affect GH release (Pombo *et al.*, 2000). Efficient GHRH transcription is sensitive to Ikaros (Ezzat *et al.*, 2006), Gsh-1, and CREB (Mutsuga *et al.*, 2001). Testing the hypothesis that hypothalamic *Rasgrf1* expression could be influencing *Ghrh* transcription and subsequent axis functioning, we quantified the amount of *Rasgrf1* present in the hypothalamus of P21 male mice (**Figure II.6**). Nulls significantly underexpressed *Rasgrf1* relative to biallelic mice ($p<0.0001$), monoallelic-paternal mice ($p<0.0001$), and monoallelic-maternal mice P=0.0006). This pattern of expression follows the trends in IGF-1 levels, both at protein and transcript level, with the nulls expressing significantly less *Rasgrf1* than the other three genotypes. The correlation between the pattern of *Rasgrf1* expression in hypothalamus and circulating IGF-1 protein suggests that GH/IGF-1 axis functioning is affected by relative amounts of *Rasgrf1* at its

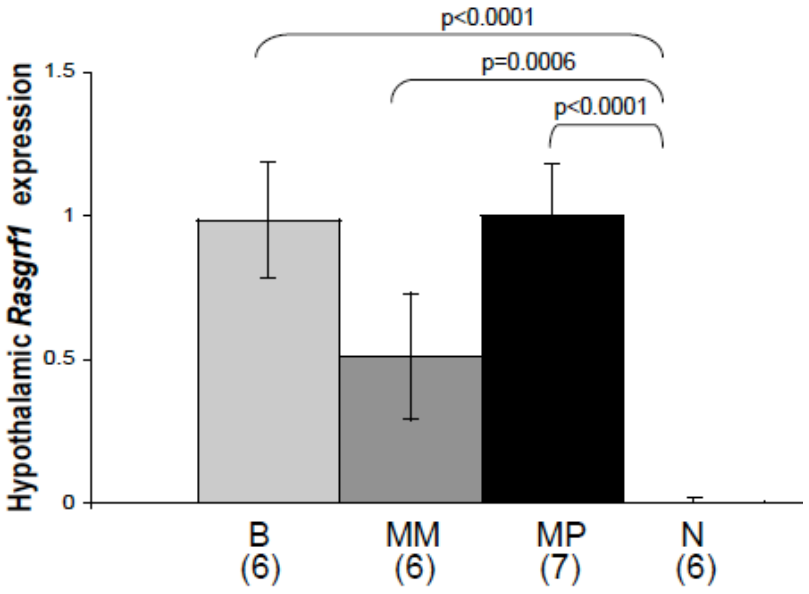


FIGURE II.6: Hypothalamic *Rasgrf1* Expression at P21 reflects trends in IGF-1 levels. Differences in hypothalamic *Rasgrf1* expression level mirror trends in weight, IGF-1 level, and axis components. *Rasgrf1* was quantified in P21 male hypothalamus using a Taqman© probe specific for *Rasgrf1* and normalized to 18S rRNA. Expression is plotted relative to wild-type animals, with the number of animals indicated; error bars represent standard errors. Null mice significantly underexpress *Rasgrf1* relative to biallelic mice ($p<0.0001$), monoallelic-paternal mice ($p<0.0001$), and monoallelic-maternal mice ($p=0.0006$).

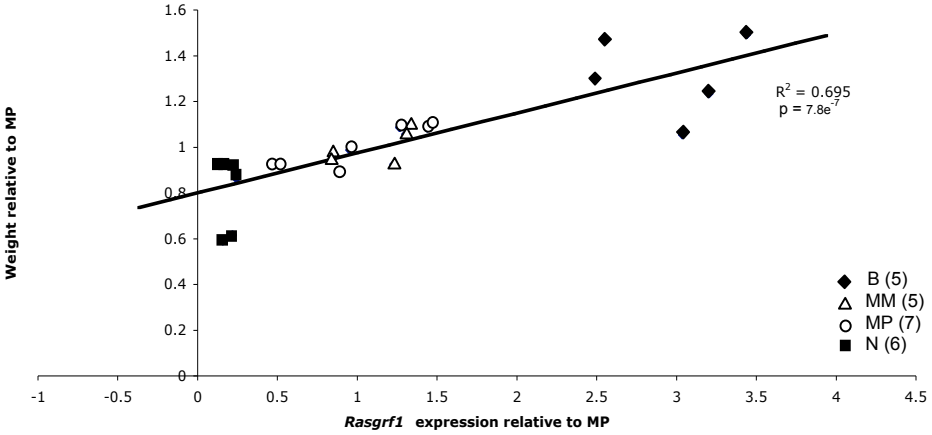
hypothalamic input, which ultimately affects levels of several genes regulated by target transcription factors that can affect IGF-1 production in the liver. Curiously, significant correlations were identified in P21 male mice between relative *Rasgrf1* expression in hippocampus (p=0.0191) and olfactory bulb ($p=7.8e^{-7}$) and relative body weight (**Supplementary Figure II.S5a,b**). *Rasgrf1* expression levels in hypothalamus did not significantly correlate with body weight.

II.5 Discussion

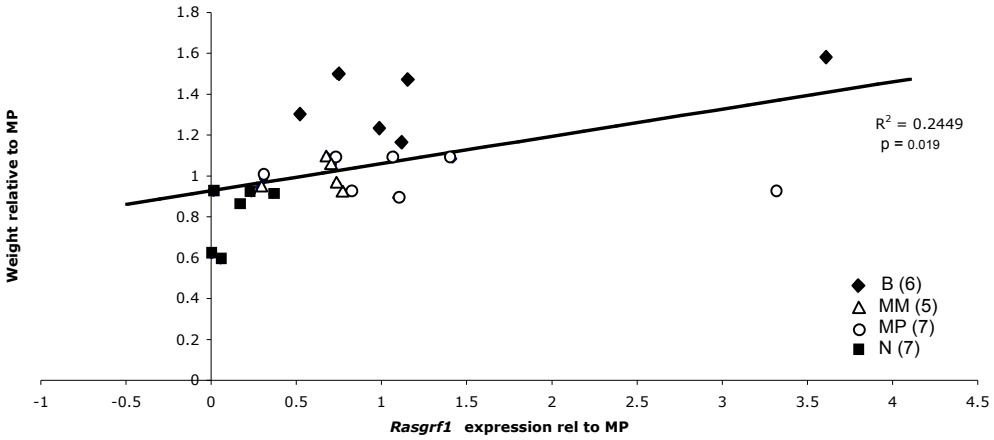
II.5.1 Weight data

The trends in size differences demonstrate two important points: that *Rasgrf1* expression levels between birth and weaning – when *Rasgrf1* shows imprinted expression – are critical determinants of body size through adulthood, and that equivalent phenotypes are produced when equivalent amounts of expression are generated from either parental allele at this imprinted locus. That the contribution of neonatal paternal allele-derived *Rasgrf1* is necessary for normal development has been demonstrated in work using *Rasgrf1* complete null alleles, where the mutant allele is inherited either maternally or paternally (Clapcott *et al.*, 2003). Maternal inheritance of a permanently null allele produces no phenotype, suggesting that the absence of maternal allele expression in both neonates and adults is not critical for normal development. Conversely, paternal inheritance of a permanently-inactivating null mutation produces a reduction in weight similar to that observed in our transient nulls, and suggests that normal activity of the

A Olfactory Bulb



B Hippocampus



SUPPLEMENTARY FIGURE II.S5: *Rasgrf1* expression level in hippocampus and olfactory bulb are correlated with body weight in P21 male animals. Weight relative to MP was plotted as a function of Rasgrf1 expression relative to MP in P21 male mice, for individual brain structures. Linear regression analysis revealed significant correlations (p-values indicated) for expression in both olfactory bulb (a) and hippocampus (b), and associated body weight. Correlations for hypothalamus and pituitary were not significant.

maternal allele in adulthood is insufficient to rescue the loss of neonatal paternal allele expression.

Thus, we can conclude that the weight differences observed in our animals are sensitive to the relative levels of pre-weaning *Rasgrf1*, which is when imprinting occurs and expression levels appear to be critical. It does appear that the engineered *tm2* allele produces a noticeable – but statistically insignificant – difference in *Rasgrf1* expression level, both in whole brain at P11 and hypothalamus at P21. Lack of statistical significance may be due to small sample size, and if this is indeed the case, it indicates that the GH-IGF-1 axis is tolerant of small differences in *Rasgrf1* expression level, such that equivalent size and level of IGF-1 are still characteristic of the two monoallelically-expressing genotypes. Larger perturbations in the expression level of *Rasgrf1* produce significant differences in size and IGF-1 levels, as evidenced by the null and biallelic animals.

Secondly, the equivalence of the monoallelic maternal and paternal phenotypes with respect to size and growth provides novel and valuable support for the conflict hypothesis describing the evolution of genomic imprinting in mammals. Because the two monoallelically-expressing genotypes exhibited identical size phenotypes, our data validates the central assumption underlying the conflict model, that growth-controlling functions of an imprinted gene are dependent only on levels of expression, regardless of the parental allele from which expression is derived. The experimental support for the conflict model provided by our engineered imprinting inversion at *Rasgrf1* could not emerge from studies of currently-existing loss-

or gain-of-function mutations at other imprinted loci. However, support for the predictions the hypothesis affords has been derived from other experimental systems, most notably for the reciprocally-imprinted *Igf2* and *Igf2r* genes, which act together in a single system to modulate expression levels and produce offspring of optimal size for both parental genomes (Filson *et al.*, 1993; reviewed by Smith *et al.*, 2006).

II.5.2 Proportional Differences in Size

Differences in size are proportional, meaning that overall size corresponds to overall weight. In the absence of GH/IGF-1 axis involvement, these data suggest that we might be seeing the effects of variations in metabolism or feeding behavior. To rule out these possibilities, we assayed the body composition of male and female mice from each of the four genotypes, and found that no significant differences existed that were consistent between the sexes. Feeding behavior is difficult to assay accurately in neonates, when animals are still nursing. Because size differences still existed in adult animals, we reasoned that feeding differences might still be detectable in adults, however our data indicate that a difference in food intake is not responsible for overall difference in body size.

II.5.3 GH-IGF-1 axis involvement

The trend toward reduced IGF-1 levels in the smaller, transient null animals, and elevated IGF-1 levels in the larger biallelic animals is clear. Serum IGF-1 and liver *Igf-1* transcript quantification both reflect this relationship. Transcript-level analysis also identifies a significant reduction in mRNA accumulation in transient null livers relative to the two monoallelic

genotypes. These data identify the GH-IGF-1 axis as an effector of misimprinted *Rasgrf1* expression on growth.

GH-IGF-1 axis components are essential for normal postnatal growth and development. We observed size differences as early as the second week of life -- earlier than that observed in GH or GH-Receptor knock-out models (Zhou *et al.*, 1997; Meyer *et al.*, 2004), which suggests that *Rasgrf1* is affecting the release and activity of IGF-1 at least preliminarily in a GH-independent manner. IGF-1 and IGF-1 receptor knock-out mice exhibit differences in size at birth, yet their phenotypes are much more severe, with a body weight 45-60% that of wild-type, and a host of other characteristics including infertility and increased rates of perinatal lethality (Liu *et al.*, 1993, 1998). That we have generated a transient imprinting mutant that doesn't fully eliminate IGF-1 expression explains the less severe phenotype we observe. GHRH knockout mice display reductions in size detectable as early as 2 weeks after birth, reduced IGF-1 in circulation, and reduced pituitary growth hormone levels (Alba & Salvatori, 2004), similar to our transient null mice. We also note that differences in pre-weaning *Rasgrf1* imprinted expression levels affect growth into adulthood, which is expected if GH-IGF-1 activity levels are established early in development and maintained through maturation.

II.5.4 IGF-1 & *Rasgrf1*

No clear link has been identified between IGF-1 and *Rasgrf1*, to our knowledge, though prior to this study there were suggestions that one influences the other. Similar to our transient null phenotype, Itier *et al.* (1997) identified reduced body weights in complete *Rasgrf1* null mice that were

accompanied by a reduction in circulating IGF-1 levels (32-40% reduced) at 4 weeks of age, and a decrease in pituitary growth hormone (42-53%) at several ages assayed. We have identified both of these characteristics in P21 transient nulls. A second suggestion of a link between Igf-1 and *Rasgrf1* is derived from data gathered using pancreatic ß-cells from *Rasgrf1* null animals (Font de Mora *et al.*, 2003). In wild-type islets, IGF-1 stimulation activates Akt and Erk, but it is not effective in stimulating signaling in islets derived from *Rasgrf1* null animals, suggesting that IGF-1 signaling is mediated by *Rasgrf1*. However, there have been no reports of *Rasgrf1* association with IGF-1 receptors, and our data suggest that the directionality of the relationship between *Rasgrf1* and IGF-1 is opposite that above, with a lack of *Rasgrf1* expression leading to a decrease in IGF-1, though IGF-1 signaling relies on feedback pathways.

We have demonstrated that feedback loops appear to be intact in our animals by the detection of somatostatin (*Sst*) overexpression in biallelic hypothalamus. Somatostatin is an inhibitor of GH release and its expression is stimulated by proper GH/IGF-1 axis function (Muller *et al.*, 1999). Thus, we would expect to see increased levels of *Sst* in response to increased levels of IGF-1.

One potential pathway connecting *Rasgrf1* to IGF-1 involves the Ikaros-dependent transcription of hypothalamic GHRH. Ikaros is an epigenetically-regulated transcription factor originally identified to play an important role in hematopoietic differentation, and it exists in 8 different isoforms (Molnar *et al.*, 1996; Molnar & Georgeopoulos, 1994). Ikaros-deficient mice share phenotypic

similarities with our transient null mice and with GHRH null mice, including a pre-weaning reduction in size, reductions in circulating IGF-1, smaller pituitary glands, and a decrease in the amount of hypothalamic GHRH (Ezzat *et al.*, 2006). Importantly, heterozygote-deficient animals display a less severe phenotype than homozygous null animals. Thus, one possibility is that Ikaros itself is a target for *Rasgrf1* signaling and is sensitive to reductions in *Rasgrf1* expression, which produces the reduction in hypothalamic GHRH in our transient nulls.

Another potential connection between *Rasgrf1* and IGF-1 is illuminated by mice that are null for Neurofibromatosis-1 (NF1) – a Ras-GAP that catalyzes the opposite reaction as *Rasgrf1*. These mice also display a phenotype similar to both the Ikaros-deficient mice and our transient null mice, with reductions in body weight, reduced hypothalamic GHRH, pituitary GH, and liver IGF-1 (Hegedus *et al.*, 2008). This phenotype is due to the Ras-independent loss of intracellular cAMP levels, a known factor in pathways leading to CREB activation, which is necessary for efficient GHRH transcription (Mutsuga *et al.*, 2001). CREB is a of RasGRF1-mediated Ras pathways, which supports the hypothesis that differences in *Rasgrf1* expression may be affecting the transcription of axis components and producing the observed reductions in somatostatin and GHRH in our transient nulls.

That the severity of the phenotype observed here is lesser than in previous work is explained by the transient effect on *Rasgrf1* expression. We are working with a gentler perturbation of expression during a specific

developmental period, which suggests again that *Rasgrf1* expression pre-weaning is critical for normal growth and development, with clear consequences that persist through maturation.

The present study has expanded on these relationships by identifying more specifically where in the GH/IGF-1 pathway *Rasgrf1* has an input. The precise mechanism by which *Rasgrf1* affects GHRH levels is still unclear; it could be via an effect on Ikaros, CREB, or Gsh-1 transcription factor activity, or an effect on neuronal connectivity between the structures responsible for the brain-derived portion of the neuroendocrine GH/IGF-1 axis, perhaps through *Rasgrf1*'s mediation of Rac signaling, which is known to play an important role in neurite outgrowth and extension (Baldassa *et al.*, 2007).

II.5.5 Concluding Remarks

We have demonstrated that normal growth is dependent on the presence of *Rasgrf1*, and that body size is sensitive to neonatal *Rasgrf1* in a dose-dependent manner; furthermore, this sensitivity does not depend on the transcribed parental allele, which provides valuable and novel support for the conflict hypothesis describing genomic imprinting in mice. We also demonstrated that mice null for *Rasgrf1* expression as neonates display a decrease in IGF-1, both in circulating protein and in liver transcript. Additionally, we demonstrate that the loss of *Rasgrf1* affects GH/IGF-1 components in structures as upstream as the hypothalamus, where we identify a decrease in *Ghrh* transcript levels. Overall, our data indicate that IGF-1 is a target for signaling pathways downstream of *Rasgrf1*, and that pre-

weaning imprinted expression of *Rasgrf1* is critical for proper sustained growth and development.

Acknowledgments: Loren DeVito for her initial observations; Dr. Yves Boisclair and Ruqian Zhao for helpful discussions and input; Mouse Metabolic Phenotyping Centers at University of Cincinnati and Vanderbilt University. Funding was from Cornell University Presidential Genomics Fellowship, NIH Training Grant T32DK007158 to Cornell University, Division of Nutritional Sciences and R01CA098597 to PDS.

III. IMPRINTED *RASGRF1* EXPRESSION IN NEONATAL MICE IS CRITICAL FOR LEARNING AND MEMORY[2]

III.1 Abstract

Rasgrf1 is imprinted and paternally-expressed in neonatal mouse brain; at weaning, expression becomes biallelic. Neonatal mice with mutations at *Rasgrf1* that confer various patterns of neonatal imprinted expression perform differently on an associative odor learning task, with poor performance corresponding to a loss of wild-type paternal allele expression. When a wild-type paternal allele is present, augmentation of *Rasgrf1* by maternal allele activation does not improve performance on the task. Various amounts of *Rasgrf1* underexpression (25% reduction to full null) lead to an inability to perform well. Differences in learning and memory among the genotypes are not present in adult mice, all of which biallelically express *Rasgrf1*. We also identify the presence of imprinted *Rasgrf1* transcript in the olfactory bulbs of neonatal mice who are otherwise null for *Rasgrf1* in the rest of the brain, and have identified alterations in the levels of active Ras and Rac proteins in brain structures related to olfactory associative learning. These results indicate that proper levels of *Rasgrf1* expression are required for learning and memory in neonatal mice, and that canonical signaling pathways are involved in such processes.

[2] Drake NM, DeVito LM, Cleland TA, and Soloway PD. In prep, for submission to *Genes, Brain, and Behavior*.

III.2 Introduction

Rasgrf1 is an imprinted gene that is paternally-expressed in neonatal mouse brain; at weaning, expression becomes biallelic (Drake *et al.*, 2009; Plass *et al.*, 1996). *Rasgrf1* is highly expressed in neurons in the central nervous system (Sturani *et al.*, 1997; Zippel *et al.*, 1997), though not exclusively so, as expression has been identified in other somatic tissues (Font de Mora *et al.*, 2003; Plass *et al.*, 1996). RasGRF1 acts as a guanine-nucleotide exchange factor for Ras and Rac proteins (Cen *et al.*, 1993; Innocenti *et al.*, 1999) by catalyzing the exchange of inactive, bound GDP for activating GTP, primarily in response to cellular calcium influx (Farnsworth *et al.*, 1995) or serine phosphorylation (Mattingly *et al.*, 1999; Yang *et al.*, 2003) in pathways downstream of muscarinic receptor activity (Mattingly & Macara, 1996), heterotrimeric G-protein subunit dissociation (Shou *et al.*, 1995; Kiyono *et al.*, 1999), and neurotrophin binding to Trk A, B, and C receptors (Macdonald *et al.*, 1999; Robinson *et al.*, 2005).

Imprinting at *Rasgrf1* is controlled by a binary switch consisting of a differentially-methylated domain (DMD) and a series of repeats immediately 3′ of the DMD . The repeats direct the placement of methylation on the paternal DMD, which at the *Rasgrf1* locus, leads to gene transcription. The maternal DMD is unmethylated, which results in CTCF (a methylation-sensitive, enhancer-blocking protein) binding; CTCF inhibits gene transcription from the maternal allele (Yoon *et al.*, 2002; Yoon *et al.*, 2005). The repeats and DMD have been manipulated to produce two engineered alleles (termed *tm1* and *tm2*), that in combination, can produce varying patterns of imprinted expression in neonatal mouse brain (Yoon *et al.*, 2002; Yoon *et al.*,

2005). In addition to the wild-type genotype (monoallelic-paternal expression, termed "MP" or "+/+"), animals have been generated that are biallelic as neonates ("B," "*tm2*/+"), null for *Rasgrf1* expression as neonates ("N," "+/*tm1*"), or reciprocally-monoallelic (monoallelic-maternal, "MM," or "*tm2*/*tm1*"). Each of these genotypes exhibits proper biallelic expression commencing around weaning, which facilitates assaying the phenotypic consequences of *Rasgrf1* imprinting perturbations specifically in neonatal mice, as well as querying the neonatal contribution to phenotypes present in adults (Drake *et al.*, 2009).

Prior work has demonstrated that *Rasgrf1* expression plays a role in postnatal growth, as *Rasgrf1* knock-out mice are smaller than their wild-type littermates (Itier *et al.*, 1998; Clapcott *et al.*, 2003). We have shown that proper imprinting in neonates is critical for maintaining a wild-type growth pattern (Drake *et al.*, 2009), an experiment that was made possible by the use of the *tm1* and *tm2* alleles. Additionally, we demonstrated that the parent from which *Rasgrf1* expression is derived does not contribute to phenotypic differences, a critical but previously untested assumption of the "conflict hypothesis" which describes the evolution of genomic imprinting in mammals (Moore & Haig, 1991; Wilkins & Haig, 2003).

Prior work has also demonstrated that *Rasgrf1* plays a role in learning and memory (Brambilla *et al.*, 1997; Giese *et al.*, 2001), as adult *Rasgrf1* knock-out mice perform poorly on behavioral assays relative to wild-type controls. However, these studies employed permanently-inactivating null alleles and assayed adult animals, which does not address the role of neonatal imprinted

Rasgrf1 expression in learning and memory. Using the *tm1* and *tm2* alleles, we can ask what the consequences of transiently perturbing *Rasgrf1* imprinting are, during the developmental period when *Rasgrf1* expression is normally imprinted.

III. 3 Materials and Methods

III.3.1 Subjects

Mice used for neonatal behavioral work were derived from two crosses: +/*tm1* males were bred with *tm2*/+ females as before, which generates four genotypes (B, MM, MP, and N) and facilitates the use of littermate controls (Yoon *et al.*, 2002; Yoon *et al.*, 2005; Drake *et al.*, 2009); +/+ females were crossed with *tm2*/+ males, which generates the +/*tm2* genotype as well as +/+ littermates to use as controls. *tm1* and *tm2* alleles are maintained on a C57BL/6 background. Mice used for RNA quantification were derived by crossing *tm2*/*tm2* , *tm1*/*tm1*, and +/+ homozygotes to generate the five genotypes (*tm2*/+, *tm2*/*tm1*, +/*tm1*, +/+, and +/*tm2*).

III.3.2 Behavioral Assay: Odors, Training and Testing

III.3.2.1 Neonatal mice, learned odor preference

Odors were prepared as in Armstrong *et al.*, 2006, with the exception that furyl-methyl ketone (FMK) and n-hexyl acetate (HA) were used. Odorants used for scenting and testing were diluted in mineral oil to liquid-phase concentrations that emitted vapor-phase partial pressures of 5.0 Pa. Behavioral assays were conducted with all mice in a given litter trained on the

same odorant, which facilitates the use of littermate controls, and rewarded odorants were balanced across litters to minimized the effects of any innate preference for either odor.

Behavior testing utilized protocols established in Armstrong *et al.*, 2006. Neonates in a litter received foot tattoos on P2. Beginning on P3, pups were separated from the dam for 90 minutes, during which they were placed on a slide warmer to help maintain body temperature. The dam's nipples were scented with the rewarded odor, and she was replaced after the 2-hour duration. Rewarded odorants were balanced across litters, with half the litters rewarded for FMK, and other half for HA.

This training procedure was repeated daily through P8, when testing occurred. To assay acquired odor preference, pups were placed in the testing chamber 3 hours after replacement of the dam and tested for place preference over the two testing odors, which were labeled "A" and "B" to facilitate experimenter objectivity. Pups were individually tested for 120 seconds, with the total time spent over the rewarded and neutral odors tallied. At the start of the testing session, pups were placed with their muzzle over the midline of the two testing compartments, and were scored as being on one side or the other when their muzzle was completely over one compartment. To control for relative immobility of pups at this age, they were replaced in the center of the testing chamber when one of the following criteria were met: a pup fell over and was unable to right itself, it reached the wall of the testing chamber, remained immobile with no head movements for 3 seconds, or began circling (more than one complete circle). Time spent over each of the two

compartments was measured by dual stopwatches, with a count-down timer set for the two-minute duration.

The testing chamber consisted of a 32x19 cm arena with a wire mesh bottom (13-cm wall height) set over two 12x19x7-cm deep compartments (Alleva & Calamandrei, 1986; Bouslama *et al.,* 2005), one of which contained Kimwipes saturated with 500ul of the rewarded odor, and one of which contained Kimwipes saturated with 500ul of the neutral odor. The two odor compartments were separated by a 7-cm wall thickness that was centrally located in the middle of the testing chamber. Between pups, placement of the compartments was switched; additionally, the wire mesh was reoriented with respect to room placement halfway through each litter to control for exogenous environmental cues. Odor compartments were cleaned with ethanol and allowed to dry between testing sessions.

III.3.2.2 Neonatal Mice, innate odor preference

Innate odor preference testing consisted of the place-preference portion of the learned odor preference paradigm, minus the training sessions. P8 neonates were placed in the testing chamber with the two training odors for a 2-minute duration. As before, dual stopwatches recorded time spent over each of the odors, with a countdown timer measuring the 120-second time period. Preference for one odor or the other was indicated by the amount of time spent over each of the odor compartments.

III.3.2.3 Juvenile Mice, passive avoidance

Juvenile mice were trained using a passive-avoidance foot shock paradigm, in which a shock was administered upon stepping down off a platform in the testing chamber. Latency to step down was measured at 30min, 24-hour, and 72-hour intervals.

III.3.2.4 Adult Mice

Adult mice were tested on a variety of parameters related to motor skills and coordination, learning and memory, and exploratory behavior (Thom Cleland).

III.3.3 *Rasgrf1* Quantification

Rasgrf1 quantification was carried out as before (Drake *et al.*, 2009). Briefly, brains were collected from P8 neonates, and relevant structures (hippocampus and olfactory bulbs) were dissected out. RNA was extracted, reverse-transcribed, and quantified in triplicate using an ABI Taqman© probe specific for *Rasgrf1*; 18s rRNA was used as a control to which *Rasgrf1* levels were normalized.

III.3.4 *Rasgrf1* Imprinted Expression

RNA was isolated from P8 neonatal brains. Mice arose from reciprocal crosses between C57Bl/6 and FvB/N males and females. RNA was reverse transcribed into cDNA, and PCR amplified with the following primers and cycling conditions: (F) 5′-ggctcatgatgaatgccttt-3′ (R) 5′-tacagaagcttggcgttgtg-3′; annealing at 58C x 40 cycles. PCR products were then digested with 10U *Ac*iI, which recognizes a restriction site (C′CGC) in exon 14 that distinguishes

expression derived from either of the two parental strains. C57BL/6-derived expression is indicated by bands of 210/146bp, and FvB/N-derived expression is indicated by bands at 226/130bp.

III.3.5 Ras and Rac activation assays

Brains were extracted, and relevant structures (hippocampus, olfactory bulbs, and cortex) were dissected and placed in Krebs-Ringer solution (11.1mM glucose, 1.1mM $MgCl_2$, 1mM Na_2HPO_4, 1.3mM $CaCl_2$, 25mM $NaHCO_3$, 120mM NaCl, 4.7mM KCl). Protein was extracted on ice. Extraction buffer included 1mM sodium orthovanadate (Na_2VO_4), 25mM sodium fluoride (NaF), and EDTA-free protease inhibitor tablets (Roche) in magnesium-containing buffer supplied by Millipore (#20-168; 125mM HEPES, pH 7.5, 750mM NaCl, 5% Igepal CA-630, 50mM $MgCl_2$, 5mM EDTA, and 10% glycerol). Extracts were affinity purified using Ras (17-218) and Rac (17-283) activation assay kits supplied by Millipore. Proteins were SDS-PAGE electrophoresed, blotted, and probed using α-Ras (05-516) and α-Rac (05-389) antibodies supplied with the kit in conjunction with goat α mouse HRP-conjugated IgG secondary antibody from Millipore (12-349). Blots were visualized using Pierce SuperSignal West Dura Substrate (34075), captured using the LAS-4000 imager's CCD camera and chemiluminescent detection function, and quantified using Multigauge Software.

III.3.6 Statistics

Expression, behavior, and protein data were analyzed using the student's *t*-test or the nonparametric Mann-Whitney U-test, and p-values were Bonferroni-corrected to account for multiple comparisons.

III.4 Results

III.4.1 Behavioral Assays

III.4.1.1 Neonates

Given that *Rasgrf1* expression is imprinted only in neonatal mice, we asked whether neonatal learning and memory might be affected by imprinting perturbations at the locus. To do this, we employed an associative odor-learning paradigm suitable for use in neonates (Armstrong *et al*., 2006), in which neonatal mice were trained to learn and remember an introduced maternal odor over a five-day training period. Neonates were tested for learned odor preference on P8. We studied five genotypes: biallelics (B), nulls (N), monoallellic-paternal (MP; wt), monoallelic-maternal (MM), and a fifth genotype with *tm2*-derived paternal *Rasgrf1* expression (+/*tm2*). This last genotype functioned to control for any differences in expression produced by the *tm2* allele, which is the sole source of *Rasgrf1* expression in the MM neonates.

As the associative odor learning paradigm asks whether neonatal mice can remember and distinguish between two testing odorants (n-hexyl acetate ["HA"] and furyl methyl ketone ["FMK"]), we first assayed innate odor preferences among the five genotypes using the place preference portion of the learning paradigm at postnatal day 8 (**Figure III.1**).

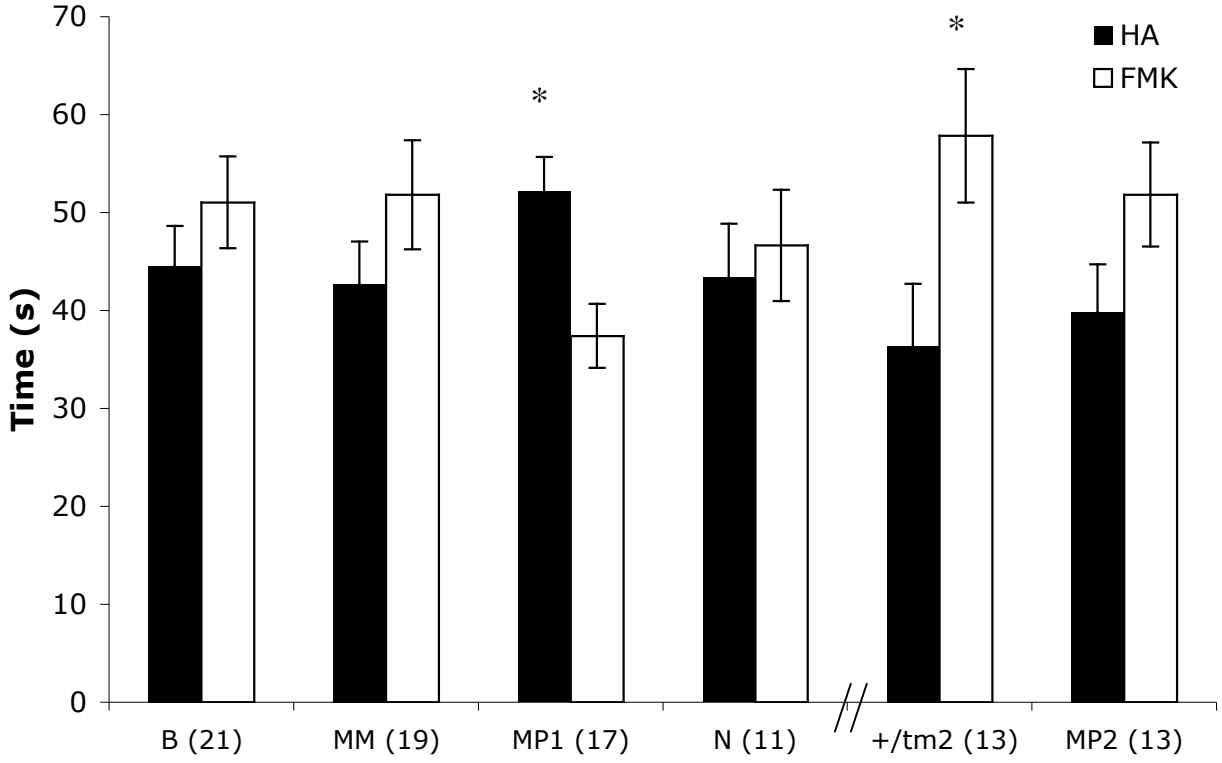


FIGURE III.1. **Innate odor preference in P8 neonates.** Neonatal mice were tested for any innate odor preference for either HA or FMK by using the place preference portion of the associative odor learning assay, minus the training portion. Mice were allowed to indicate a preference for a 120-sec duration, and times spent over each odor are plotted. The numbers of mice tested are indicated in parenthesis next to genotype. Asterisks indicate p-values <0.05. A significant preference was detected in the *+/tm2* genotype; any significant difference in the wild-type mice disappears when the data for that genotype (M1 and MP2) are combined.

Neonates were placed in the tesing chamber for a 120-second duration. Interestingly, the only genotype consistently indicating a preference for either of the two testing odors was the +/*tm2* genotype (n=13; p=0.02). B (n=21; p=0.30), MM (n=19; p=0.30), and N (n=11, p=0.59) animals displayed no preference for either odor. The two MP cohorts (one produced from the breeding scheme generating the B, MM, and N animals [MP1], and the other from the breeding scheme producing the +/*tm2* animals [MP2]) varied in their preferences. MP1 animals (n=17, p=0.0041) displayed a preference for HA; MP2 animals (n=13; p=0.11) displayed no preference. When the two MP cohorts are combined, no statistically significant preference is detected (n=30; p=0.47).

HA and FMK were selected for use in the learned odor preference paradigm, as the four main genotypes displayed no preference for either, including the MM animals, where *Rasgrf1* expression is *tm2*-derived. The 'rewarded' odorant was counterbalanced across litters, with half the litters trained to recognize HA, and the other half trained to recognize FMK.

Learned odor preferences (**Figure III.2**) were detected in the two genotypes with paternally-inherited wild-type alleles, the biallelics (n=59, p=0.0073) and the wild-types (MP1: n=53, p=0.0002; MP2: n=15, p=0.0068). There was no significant enhancement in preference among biallelic pups relative to wild-type littermates, as differences in time spent over each odor were statistically insignificant between genotypes (p=0.62). Unlike the B and MP animals, the other three genotypes did not demonstrate a similar ability to learn and remember an introduced maternal odor, as they exhibited no

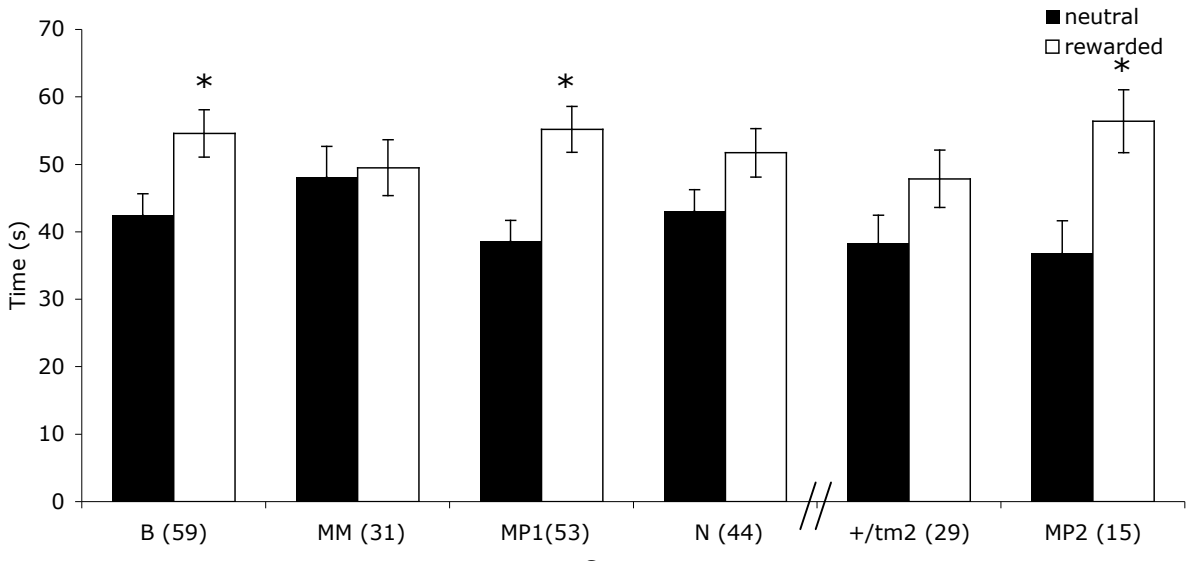


FIGURE III.2. **Learned odor preference in P8 neonates.** Neonatal mice were tested for for their ability to learn and remember an introduced maternal odor. Mice were tested for place preference over either the neutral or rewarded odor for a 120-second duration, and times spent over each are plotted. The numbers of mice tested are indicated in parenthesis next to the genotypes. Asterisks indicate p-values <0.01. Significant preferences for the rewarded odor were detected only the biallelic and wild-type cohorts.

preference for the rewarded odor relative to the neutral odor (MM, n=31, p=0.41; N, n=46, p=0.1; +/*tm2*, n=29, p=0.11).

Learned odor preference data indicates that the *tm2* allele essentially behaves like a null allele, regardless of whether it is materally- or paternally-inherited, as neither the MM nor the +/*tm2* animals indicated a preference for the rewarded odorant. This pattern was expected in the null animals (due to prior demonstrations of learning deficiencies in *Rasgrf1* -/- mice), but was surprising in the MM and +/*tm2* genotypes, which were phenotypically indistinguishable from MP animals in terms of body size and growth (Drake *et al.*, 2009), which is another phenotype that *Rasgrf1* contributes to. No significant differences in weight or length were observed between wild-type and +/*tm2* males or females (data not shown), indicating that – similarly to maternally-derived *tm2* expression – paternally-derived *tm2* expression is sufficient to produce a wild-type size phenotype, but insufficient for production of wild-type learning and memory, as assayed by the olfactory odor learning paradigm.

III.4.1.3 Juvenile mice

We assayed learning and memory in juvenile mice using a passive avoidance foot-shock paradigm in which mice were trained on P14 to avoid stepping off a platform. Latencies to step down were measured at 30min, 24-hour, and 72-hour intervals, and no significant results were obtained (data not shown). We hypothesize that this could be due to the gradual acquisition of *Rasgrf1* maternal-allele expression at this age.

III.4.1.3 Adult mice

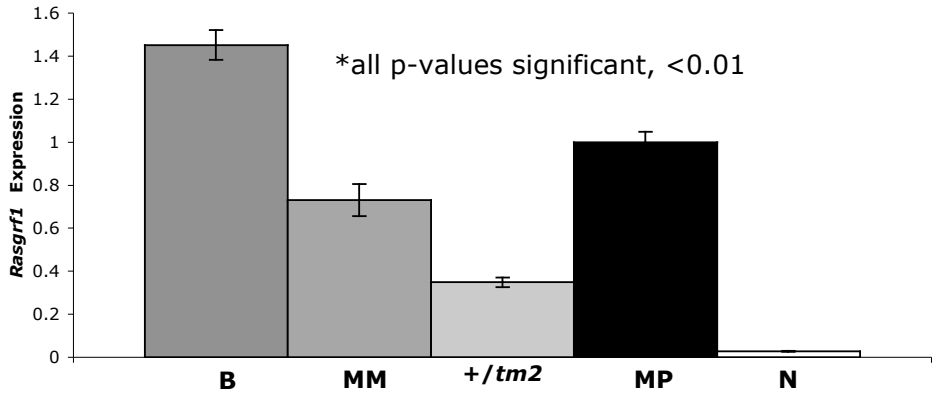
No differences in performance on any assay were identified among adult mice. We hypothesize that the lack of phenotype present in adult animals is due to the fact that all genotypes biallelically express *Rasgrf1*, thus any differences due to *Rasgrf1* expression are ameliorated at these ages, therefore eliminating any resulting behavioral phenotype.

II.4.2 Transcript Quantification

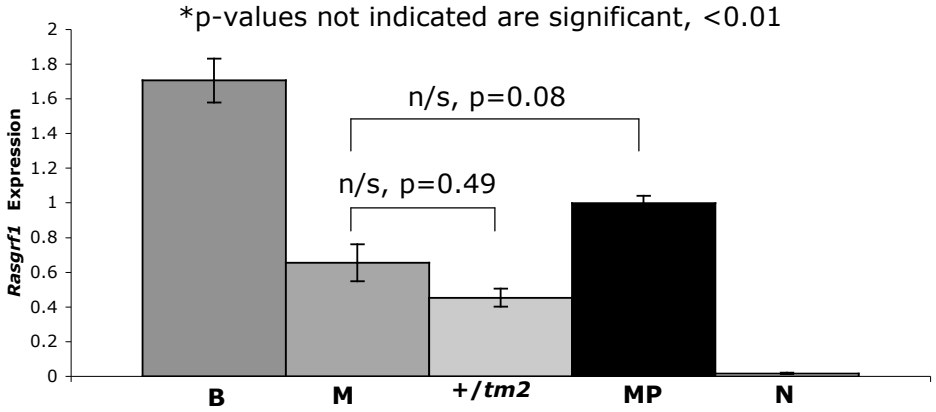
Given that the *tm2* allele essentially functions as a null allele in the learning and memory assay, we asked whether the level of *tm2*-derived *Rasgrf1* expression differed from wild-type allele expression. *Rasgrf1* transcript level was quantified in P8 neonatal olfactory bulb, hippocampus, and whole brain for each of the five genotypes using a Taqman© probe specific for *Rasgrf1*, with data normalized to 18s rRNA levels, and then further normalized to MP *Rasgrf1* expression level (**Figure III.3**). For all quantification data, statistically-significant p-values are those that are $p<0.005$, the value derived by Bonferroni-correcting for 10 multiple comparisons, and n=6 for all genotypes except for MP, where n=7. Transcript levels in brain (**Figure III.3a**) were roughly as expected ,with biallelic mice overexpressing *Rasgrf1* ($p=0.001$) and null mice underexpressing *Rasgrf1* ($p<0.001$) relative to the wild-type, MP animals. However, the MM ($p=0.0047$) and +/*tm2* ($p=<0.001$) animals were intermediate in expression level between the MP animals and nulls, and significantly different from MP, a result which differs from that obtained in P11 mice (Drake *et al*., 2009; dissertation Chapter 2, Figure II.2), but does correlate with the phenotypic pattern identified in the learning paradigm.

FIGURE III.3. ***Rasgrf1* transcript quantification in P8 brain regions.** *Rasgrf1* transcript was quantified in brain (a), hippocampus (b), and olfactory bulb (c). Values were then normalized to wild-type. N=6 animals for each genotype, except for MP (n=7). All p-values for brain (**a**) are significant and <0.01. P-values for hippocampus (b) are significant and <0.01 unless indicated. Olfactory bulb (OB) (**c**) p-values are significant and <0.01 unless indicated. OB produces quantification results mirroring the phenotypic clustering of genotypes in the learning assay: the biallelics and wild-type mice are similar, and the monoallelic-maternal, +/*tm2*, and null mice are similar. Additionally, a low but significant level of expression is detected in the null mice in this structure.

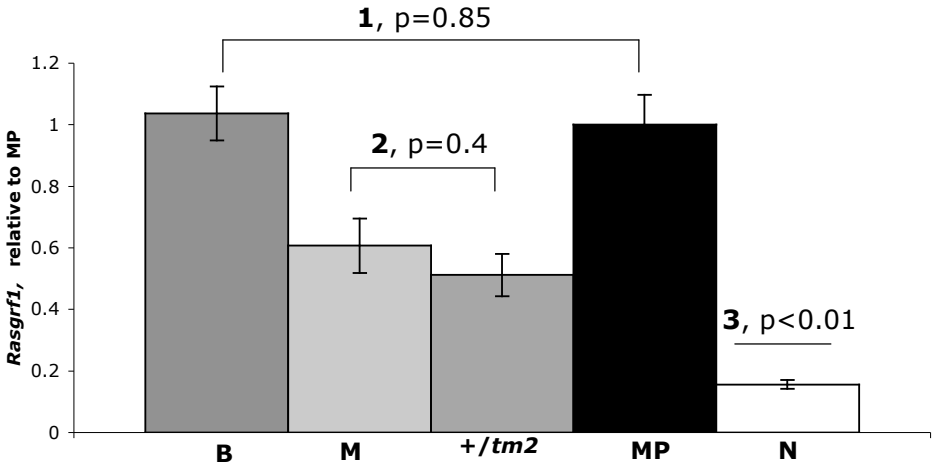
A


*all p-values significant, <0.01
Rasgrf1 Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
B
MM
+/tm2
MP
N

B


*p-values not indicated are significant, <0.01
n/s, p=0.08
n/s, p=0.49
Rasgrf1 Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
B
M
+/tm2
MP
N

C


1, p=0.85
2, p=0.4
3, p<0.01
Rasgrf1, relative to MP
1.2
1
0.8
0.6
0.4
0.2
0
B
M
+/tm2
MP
N

Additionally, in whole brain, *Rasgrf1* expression level was significantly different between the MM and +/*tm2* animals.

We reasoned that while data derived from the brain as a whole may match the phenotypic trend in learning, it may not be specific enough to indicate which structures might be involved in producing the learning and memory phenotype, so we
further assayed expression in the hippocampus and olfactory bulb, the two structures most likely involved in olfactory-based learning.

Rasgrf1 expression patterns in hippocampus (**Figure III.3b**) varied from those in brain: Biallelic (p=0.0023) mice again overexpress *Rasgrf1* relative to wild-type, null mice again underexpress *Rasgrf1* (p=<0.001) relative to wild-type, +/*tm2* (p=<0.001) animals are again intermediate between the wild-type and null levels, but the monoallelic-maternal mice (n=6, p=0.0128) are indistinguishable from wild-type animals. Furthermore, there is no significant difference between the +/*tm2* and MM genotypes in this structure (p=0.0338). With the exception of the significant difference between +/*tm2* and MP genotypes, this pattern most closely corresponds with the phenotypic trend we see in terms of body size, where biallelic animals are the biggest, null animals are the smallest, and the three monoallelically-expressing genotypes are intermediate and indistinguishable from one another.

Olfactory bulb quantification (**Figure III.3c**) produced an unexpected result. First, the biallelic and wild-type animals were not significantly different from one another at the transcript level (p=0.8515), which mirrors the

trend in behavioral data. Monoallelic-maternal and +/*tm2* animals were intermediate in expression level between the wild-types and the nulls, and again there was no significant difference between those two genotypes ($p=0.26$), as in hippocampus. The nulls were again underexpressing *Rasgrf1* relative to wild-type ($p<0.001$); however, in olfactory bulb, the 'nulls' were not null. Instead, a low but significant level of *Rasgrf1* was detected, approximately 20% that of wild-type. Though unexpected, this quantification result nicely mirrors the phenotypic trend identified in the learning and memory task: the biallelic and wild-type animals perform significantly better than the null, monoallelic-maternal, and +/*tm2* genotypes.

III.4.3 Rasgrf1 is expressed and imprinted in null olfactory bulb

PCR analysis confirms the presence of visible *Rasgrf1* bands in null olfactory bulb cDNAs (n=6) and the absence of *Rasgrf1* in null brain (n=6; **Figure III.4a)**. Biallelic brain (n=5) and olfactory bulb (n=6) cDNAs were run as controls for amplification, and *Rasgrf1* transcript was detected in both tissues. RNA alone (with no RT reaction) produced no transcript.

We next asked whether *Rasgrf1* transcript detected in olfactory bulb was imprinted, or if imprinting mechanisms in this structure were behaving differently than in whole brain. Reciprocal crosses between B6 and FvB/n animals were set up, as well as a cross between an FvB/n mother and *tm1*/*tm1* homozygous father. Olfactory bulbs from P8 brains derived from these crosses were dissected, cDNAs were generated, and *Rasgrf1* transcript was assayed for parental-specific expression using an *Aci*I restriction site allowing

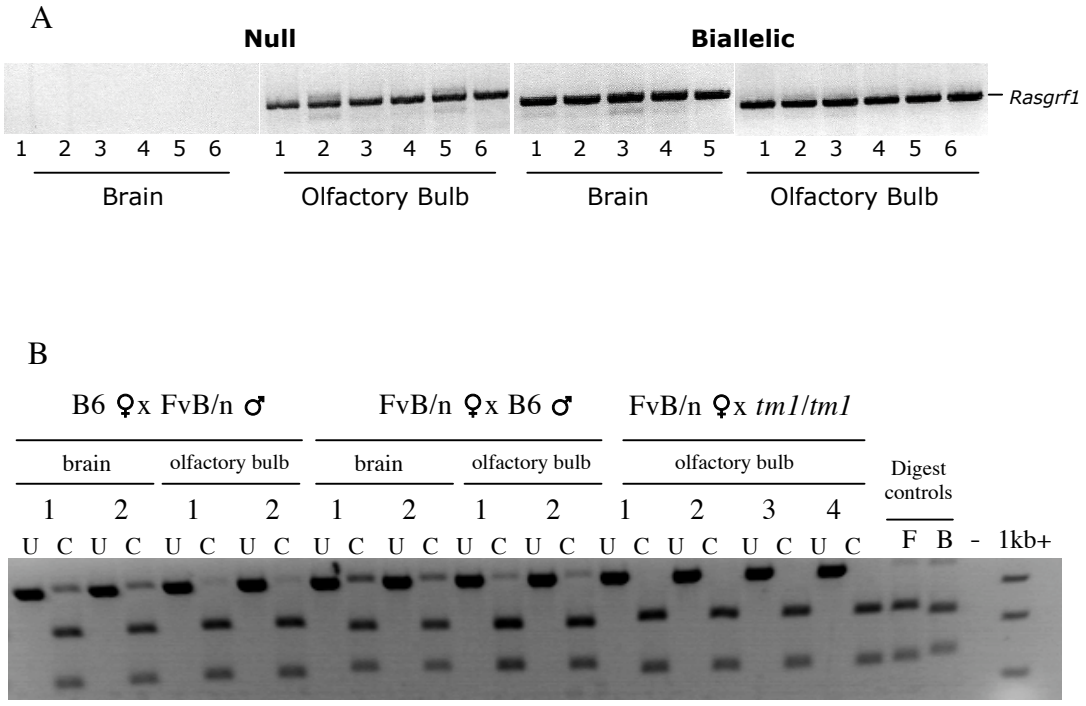


FIGURE III.4. ***Rasgrf1* is expressed and imprinted in neonatal null olfactory bulb.** *Rasgrf1* trancript was amplified in null and biallelic brains and olfactory bulb using RT-PCR (**4a**). Controls with RNA only produced no amplification, indicating that the presence of *Rasgrf1* bands in null olfactory bulb cDNAs is indicative of expression. Imprinted expression in olfactory bulb was established by PCR amplification and AciI digestion of transcripts produced by reciprocally crossing B6 (B) and FvB/n (F) parents (**4b**); an additional cross (FvB/n x *tm1/tm1)* confirmed the presence of Rasgrf1 transcript, despite the presence of two paternal *tm1* alleles. Digestion of amplicons produces exclusively paternal banding patterns. FvB/n-derived transcript yields digested band sizes of 226bp and 130bp, and B6-derived transcript yields band sizes of 210bp and 146bp.

distinction of transcript derived from each of the two strains. Results indicated that *Rasgrf1* in olfactory bulb is imprinted and paternally-derived (**Figure III.4b**) as in the rest of the brain, indicating that the paternal allele in null animals somehow escapes the silencing mechanism generated by the loss of DNA methylation at the *Rasgrf1* primary DMD (Yoon *et al.*, 2002 & 2005).

Progeny from B6 mothers and FvB/n fathers displayed 226/130bp bands characteristic of FvB/n-derived *Rasgrf1* transcript; progeny from FvB/n mothers and B6 fathers displayed the 210/146 bands characteristic of B6-derived *Rasgrf1* transcript. The *tm1* allele was created in a 129S4Jae line, and maintained on the B6 background; 129 and FvB/n strains share the same *Aci*I polymorphism, so the banding pattern following digestion does not indicate from which parent *Rasgrf1* expression in this particular cross is derived. However, the clearly monoallelic-paternal result from the two wild-type reciprocal crosses, combined with the fact that we do see *Rasgrf1* from this cross – where both paternal alleles contain the repeat-deletion – suggests that the requirement for repeat-directed DNA methylation at the *Rasgrf1* DMD is relaxed in olfactory bulb, since transcript is still produced, although at lower levels than in wild-type mice.

III.4.4 Other Approaches

Though differences in olfactory bulb *Rasgrf1* transcript level appear to explain the discrepancies in performance on the learning and memory task, we also considered whether *tm2*-derived expression varies from wild-type by cell type or overall distribution in the brain (Yoon *et al.*, 2005). Immunohistochemical approaches were ultimately unsuccessful due to the

lack of an antibody specific for RasGRF1 peptide; commercially-available antibodies also detect RasGRF2, a closely-related peptide that is expressed in brain (Fam *et al.*, 1997), making identification of differences between genotypes unreliable by this method. Additional attempts were made to visualize *Rasgrf1* expression in brain using RNA *in situ* hybridization, but probes appeared to cross-react with *Rasgrf2* as well (see dissertation Appendix V.2).

III.4.5 Signaling

RasGRF1 acts as a guanine-nucleotide exchange factor for both Ras and Rac proteins, and we were curious to know whether the activation of either of these proteins was influenced by mutations at *Rasgrf1*. Prior work indicates that RasGRF1 may not be an active signaling intermediate in neonatal mice (Tian *et al.*, 2004), but we detected differences in associative odor learning that correspond to the amount of *Rasgrf1* expressed in neonates. Additionally, those prior assays were completed using whole-brain protein extracts, and our transcript quantification work indicates that there are differences in the relative amounts of *Rasgrf1* transcript that vary by structure.

We therefore attempted to identify protein activation state using specific structures derived from P8 neonates. Because the MP and N mice were significantly different from one another, we used these two genotypes for this test. Protein extracts from brain, olfactory bulb, and hippocampus were assayed for the presence of activated Ras and Rac proteins. Activated protein was affinity-purified by incubating extracts with glutathione agarose beads to which either the c-Raf (for Ras) or PAK-1 (for Rac) protein binding

domains were fused, which facilitates interaction with the activated forms of the proteins. The amount of protein pulled down via binding was then normalized to the total amount of Ras or Rac protein in the input lanes, which allowed us to semi-quantitatively determine whether there were differences in the amount of activated protein between genotypes.

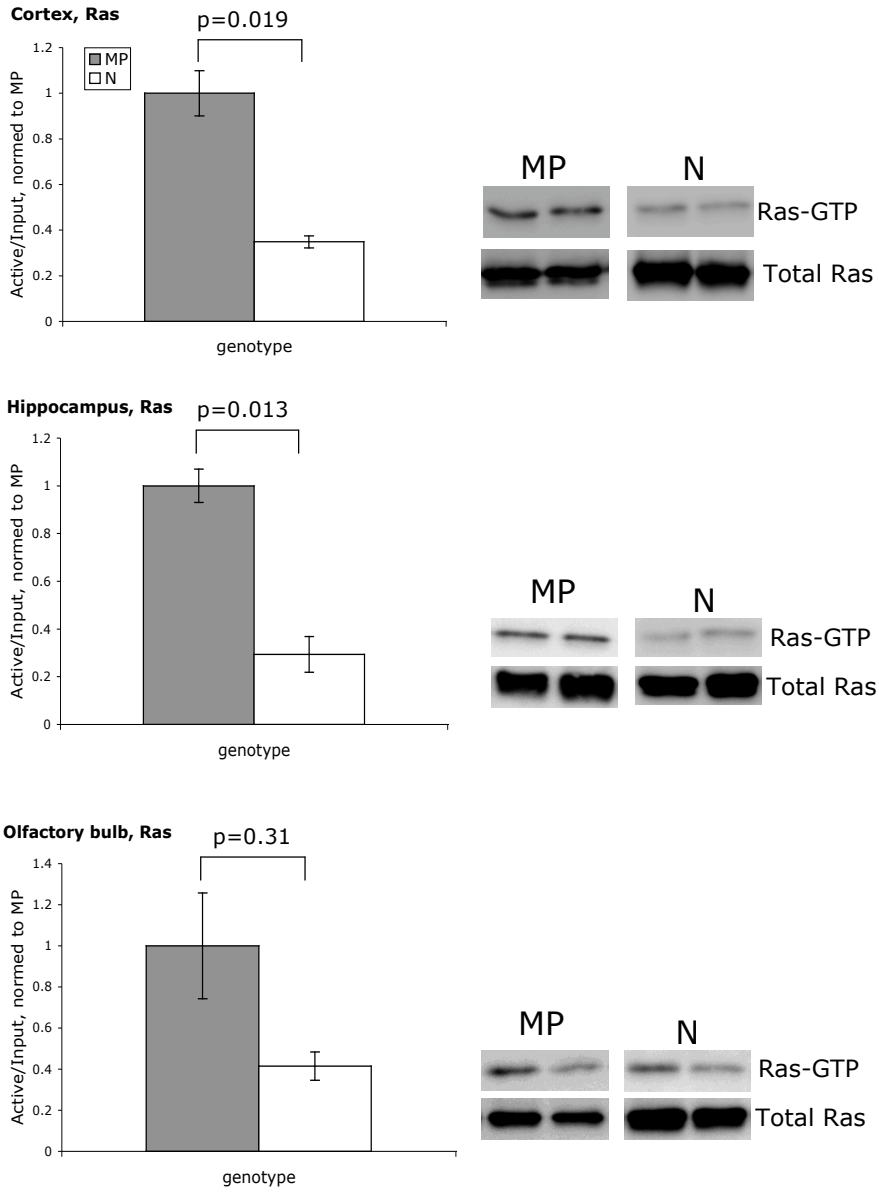
Blots indicated significant alterations in the amount of activated Ras and Rac protein between the wild-type and null animals (**Figure III.5**). Data in Figure 5 are presented as the ratio of activated protein to input, normalized to wild-type.

Cortex produced significantly higher amounts of activated Ras in the wild-type animals, relative to the nulls ($p=0.0191$), and wild-type hippocampal extracts contained a higher amount of activated Ras and Rac proteins as well ($p=0.0137$, $p=0.0474$, respectively). Oddly, the null animals display an increase in the amount of activated Rac in cortical extracts, relative to wild-type animals. Olfactory bulb extracts produced the lowest amount of activated proteins, for both Ras and Rac, which made even semi-quantitation of null Rac levels unreliable. There were no significant differences in the amount of activated Ras protein between genotypes in olfactory bulb extracts.

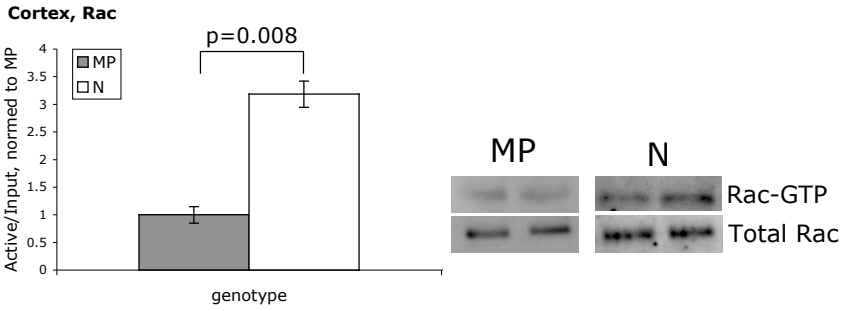
Taken together, these data indicate that there are significant differences in the amount of active signaling proteins in wild-type and null brains, and that there are structure-specific differences in relative protein activation state, which suggests that assays measuring activity might dilute differences in individual structures when using whole brain extracts.

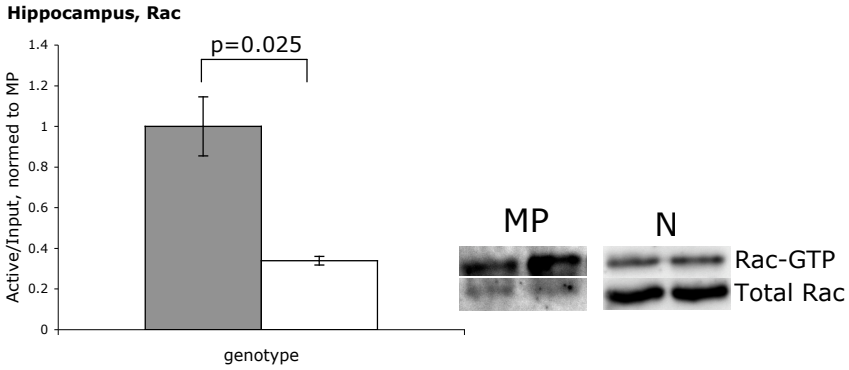
FIGURE III.5. Semi-quantitative analysis of the relative amounts of activated Ras (a) and Rac (b) proteins in null and wild-type brains regions varies by structure. The amounts of precipitated (active) protein were normalized to the amounts of input (total) protein for each structure, and then normalized to wild-type. There are reduced amounts of active Ras, relative to wild-type, in null cortex and hippocampus; activated Rac is also reduced in null hippocampus, relative to wild-type. Increases in activated Rac are seen in null cortex. n=4 for all comparisons, and p-values are indicated. Representative blots are shown next to each graph.

III.5a

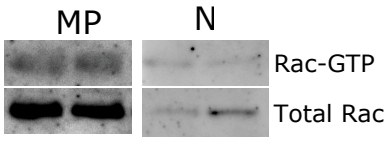

Cortex, Ras
p=0.019
MP
N
Active/Input, normed to MP
genotype
MP
N
Ras-GTP
Total Ras
Hippocampus, Ras
p=0.013
Active/Input, normed to MP
genotype
MP
N
Ras-GTP
Total Ras
Olfactory bulb, Ras
p=0.31
Active/Input, normed to MP
genotype
MP
N
Ras-GTP
Total Ras

III.5b (Continued)





Olfactory Bulb, Rac



Thus, while we have not been able to directly link the olfactory learning phenotype with a known biochemical pathway, we can conclusively identify *Rasgrf1* expression level as being critical for the production of normal olfactory associative learning, and can identify subtle but significant differences in small G-protein activation that depend on the amount of *Rasgrf1* expressed, but the dependency varies with tissue type.

III.5 Discussion

III.5.1 *Rasgrf1* expression-dependent phenotypes

This study demonstrates that neonatal learning and memory – as assayed at P8 using an associative odor-learning paradigm – is critically dependent upon proper *Rasgrf1* expression levels in neonatal brain, and is the first study to assay *Rasgrf1*-dependent neonatal learning and memory. Even a modest reduction in *Rasgrf1* level in MM mice at the whole brain level (73% that of wild-type) is enough to produce a diminished ability to learn and remember an introduced maternal odor. Interestingly, this same difference between these two monoallelic genotypes is not sufficient to produce a difference in overall growth (Drake *et al.*, 2009; see Dissertation Chapter 2, Figure II.3), suggesting that the mechanisms involved in learning and memory are much more sensitive to *Rasgrf1* level than those involved in growth; however, *Rasgrf1* does not appear to be a critical limiting factor, as overexpression in biallelics does not produce a detectable enhancement in neonatal learning.

The results of our previous study supported a previously untested assumption of the conflict hypothesis (Wilkins & Haig, 2004), which describes the evolution of genomic imprinting in mammals as ultimately being a tug of war between the two parental genomes over optimum expression level at imprinted loci (Moore & Haig, 1991). Previously untested was the assumption that equivalent amounts of expression derived from either parental allele would produce an equivalent phenotype, which we did observe with regard to the size phenotype at the ages assayed. Thus, that there are differences in phenotype between the MM and MP mice in this study does not disprove Moore & Haig's assumption, as *tm2*-derived expression at P8 does not recapitulate wild-type expression level. The results of the current study instead indicate that for proper learning and memory to occur at this age, there needs to be a level of *Rasgrf1* expressed exceeding that produced by the *tm2* allele, though we cannot rule out differences in cell type or neural distribution as contributing factors . However, the results of this study also support Moore and Haig's assumption because *tm2*-derived expression produces the same neonatal learning phenotype, regardless of whether the allele is maternally or paternally inherited.

III.5.2 *Rasgrf1* in Olfactory Bulb

Our current results also indicate that *Rasgrf1* expression in olfactory bulb may be subject to different epigenetic regulatory mechanisms than in other tissues. Null animals display *tm1*-derived expression in olfactory bulb, at a level 20% that of wild-type, which is in contrast to the 1-2% observed in hippocampus and whole brain, respectively. Expression in this structure at this age is exclusively paternally-derived, which indicates that the *tm1* repeat

deletion is either failing to produce relevant hypomethylation, or that transcription regulatory mechanisms controlling imprinted expression in the olfactory bulb differ from those used in other tissues, where imprinting also occurs.

III.5.3 Age-related phenotypes

The loss of a behavioral phenotype with age is also not surprising, as *Rasgrf1* expression gradually becomes biallelic around weaning. As is the case with the *tm1* allele escaping identified epigenetic controls, the mechanism whereby the maternal allele becomes actively transcribed with maturation is also unknown. We do observe a lack of behavioral phenotype as early as P17, which corresponds to the gradual onset of maternal allele expression (Drake *et al.*, 2009), and a similar paucity of performance differences in behavioral assays conducted using adult mice.

Our previous observations (Drake *et al.*, 2009) suggest that the production of the growth phenotype relies solely on neonatal differences in *Rasgrf1* level, which explains the persistence of size differences past the point at which expression becomes biallelic. The behavior phenotype identified in this study is different, in that it is responsive to the accumulation of maternal transcript around weaning, which leads to the diminishing of performance differences among genotypes at later ages. However, despite these differences – and indeed they suggest that *Rasgrf1* functions in different pathways subject to different regulatory mechanisms – there are similarities between the two phenotypes. The two extreme genotypes – the nulls and the biallelics – display early phenotypic differences both in size and in neonatal

learning. Transient underexpression of *Rasgrf1* in nulls leads to smaller animals with an impaired ability to learn and remember an introduced maternal odor. Transient overexpression in biallelics produces larger animals that are capable of learning and remembering an introduced maternal odor, at least as well as wild-type animals, though overexpression does not produce any enhancement in learning and memory, suggesting that *Rasgrf1* is not the rate-limiting molecule in the relevant pathway. Additionally, overexpression does not lead to learning and memory deficiencies, suggesting that excess *Rasgrf1* is not toxic to the organism. Thus, transient changes in expression do not appear to be detrimental to overall fitness in any way, as animals of all genotypes survive to reproductive age and are capable of bearing litters. That one phenotype is more sensitive to overall *Rasgrf1* expression level is the only significant difference between the two presentations, in addition to the loss of learning and memory differences that occur with aging.

III.5.4 *Rasgrf1* and Odor Learning

This work also highlights a potential role for *Rasgrf1* in early associative odor learning, as reductions in *Rasgrf1* expression produce an inability to associate odor with reward. Early olfactory associative learning involves an NMDA-receptor dependent process (Lincoln *et al.*, 1988; Weldon *et al.*, 1997), and is mediated by CREB phosphorylation (McLean *et al.*, 1999; Raineki *et al.*, 2009). The contribution of NMDA receptors to olfactory learning appears to be well-conserved, as similar processes are observed in *Drosophila* (Xia *et al.*, 2005) and honeybees (Si *et al.*, 2004), in addition to rats (Tronel & Sara, 2003) and mice (Brennan, 1994). RasGRF1 is known to directly associate with NMDA receptors, via the NR2B subunit (Krapivinsky *et al.*, 2003), which is

striking given that certain olfactory learning processes are NR2B-dependent in particular (White & Youngentob, 2004). Furthermore, *Rasgrf1* has been shown to transduce signals arriving at the NMDA receptor and activate the ERK/MAPK pathway in response (Krapivinsky *et al.*, 2003; Tian *et al.*, 2004) , which lies upstream of CREB phosphorylation. Somewhat confounding is the observation by Tian *et al.* (2004) that *Rasgrf1* does not play a significant role in NMDA-mediated signaling in neonatal mice. While we were unable to identify a direct link between *Rasgrf1* and Ras or Rac pathway activation, we were able to demonstrate that there are differences in the amounts of activated protein between wild-type and null animals.

Lastly, we have been able to point to a significant amount of variability present among different subcortical structures, both at the level of *Rasgrf1* expressed, and in the amount of activated protein. Though not surprising, this trend indicates that important data might be missed by simply assaying whole brain preparations instead of individual structures. For example, one possibility explaining the absence of an identifiable *Rasgrf1* contribution to neonatal NMDA signaling (Tian *et al.*, 2004) is that the use of cortical slices diluted out any relevant *Rasgrf1* contribution within specific structures.

Taken together, these results indicate that proper *Rasgrf1* expression is critical for neonatal performance on an associative odor-learning task and that mechanisms governing *Rasgrf1* expression and its effects on protein activation vary by tissue type. We also again observe the presence of a similar phenotype, regardless of whether *Rasgrf1* expression is maternally or

paternally derived, as evidenced by the similarities between the monoallelic maternal and +/*tm2* animals.

IV. EXPANDED DISCUSSION

The experiments detailed in these pages define a role for *Rasgrf1* in the presentation of two phenotypes: the first is related to growth and body size, and the second to learning and memory. That *Rasgrf1* expression plays a part in both of these systems is unsurprising, given established theory and literature – but the nature of its effect on these phenotypes and the inferences we can make about genomic imprinting are novel, due to the use of the *tm1* and *tm2* alleles. These alleles are unique in that they allowed us to examine the effects of *Rasgrf1* transcript derived monoallelically from either of the parental alleles, which has not been done before. We could compare monoallelic-maternal *Rasgrf1* expression with monoallelic-paternal *Rasgrf1* expression, which allowed us to examine whether the parental origin of the transcript is significant in the production of phenotypes. Additionally, the *tm1* and *tm2* alleles enabled us to ask what the effects of transient perturbations in imprinted *Rasgrf1* expression were, as expression becomes biallelic around weaning in genotypes produced by these alleles, which recapitulates the wild-type relaxation of *Rasgrf1* imprinting. This is important because we were able to determine whether imprinting during a specific period in development is critical for proper phenotype presentation, instead of asking more generally whether a permanent alteration in *Rasgrf1* expression produces a phenotype.

We first defined a role for *Rasgrf1* in the production of a size phenotype. Overall growth and body size – as animals were not only different in terms of body weight, but also body length – corresponded very closely to

the overall level of *Rasgrf1* expression measured in brain structures at P11 and at weaning. Biallelic mice overexpressing *Rasgrf1* transcript were larger, nulls were smaller, and the two monallelically-expressing genotypes – the MM and MP cohorts – were intermediate and indistinguishable from one another. These differences in size persisted past the point at which *Rasgrf1* expression becomes biallelic (weaning), and the phenotypic pattern suggested something to us that no prior published studies could have observed: not only does *Rasgrf1* appear to play a critical role in the early establishment of growth parameters, but more generally, imprinted expression at this locus appears to be the result of parental 'argument' over optimum expression level. Different levels of expression produce different phenotypes – but when the same amount of gene expression is derived either from the maternal or the paternal allele, no phenotypic difference presents. Neither of these observations could have been made previously because earlier studies of *Rasgrf1* function employed permanent null alleles, and such alleles are insufficient to delineate the effects of *Rasgrf1* in early and later life; additionally, our system of mutant alleles facilitated the production of the 'reciprocal' monoallelic genotype, a key component in determining whether expression from the two parental alleles produced phenotypic differences.

Our data suggest that the role *Rasgrf1* plays in the setting of a metabolic program occurs early in life (pre-weaning, with size differences observed as early as P8) and affects the function of the growth hormone/IGF-1 axis. *Rasgrf1* knockout mice have previously been observed to display deficiencies in various axis components, but our work is the first study to clearly identify where in the axis *Rasgrf1* has an input. We have measured differences in

GHRH levels, an axis component as far upstream from IGF-1 release as the hypothalamus – which clearly links the predominantly neuronal pattern of *Rasgrf1* expression with the further downstream systemic effects on axis function, namely the differences in circulating IGF-1 that are likely responsible for the size differences we observe. These data suggest that early life is a critical period for the setting of growth parameters that will be maintained through adulthood. Indeed, the role of imprinted genes in growth has been well established, and epigenetic modifications are therefore implicated in the same pathways. But, the role of epigenetic modifiers independent of imprinted genes in the production of phenotypes is just beginning to be understood. Epigenetic marks are by nature plastic, and are affected by such ubiquitous factors as dietary factors and environmental components, and could represent an additional layer of genomic sensitivity and enhanced adaptability. More generally, how have epigenetic marks shaped evolutionary processes in ways undetectable by genomic scans searching sequence data for signatures of selection and molecular evolution?

Our second set of data focused on the role of *Rasgrf1* in early learning and memory, using an olfactory associative odor-learning assay. Neonatal mice were tested for their ability to learn and remember an introduced maternal odor – and once again, performance on the task depended critically on the amount of *Rasgrf1* present in brain structures at P8. However, unlike the results from the growth assays, the two monoallelically-expressing genotypes behaved differently from one another. We hypothesized that this discrepancy was due to the mutant *tm2* allele's inability to completely recapitulate wild-type patterns of expression, and this was found to be true, as

tm2-derived levels of expression at P8 were significantly different from wild-type levels. However, varying the inheritance of the *tm2* allele did not produce different phenotypes – maternal and paternal *tm2* transmission produced the same discrepancy in performance during the associative odor learning task, as well as similar results in transcript quantity. This pattern indicates that once again – at least at the *Rasgrf1* locus – the level of imprinted gene expression is critical for the production of phenotypes dependent on imprinted genes – and that the "acceptable" threshold may vary by system.

The permanency of the differences in growth (persisting through adulthood) is offset by the flexibility of the system in allowing *tm2*-derived expression to sufficiently recapitulate a wild-type phenotype. The data from our learning and memory study indicate that at an age earlier than that assayed in the growth study (P8 v P11), there is a slight but significant difference in *Rasgrf1* transcript quantity between the MM and wild-type genotypes, in some brain structures. This difference disappears by P11, and is still insignificant at weaning. However, this same difference in expression level is insufficient to produce a wild-type behavioral phenotype at P8 – but unlike the growth phenotype, differences in learning and memory are not detectable among genotypes in adult animals, ie – the learning and memory deficiencies present in neonatal mice are transient, and are not observed in adults, when all genotypes biallelically express *Rasgrf1*. Perhaps the reason the learning and memory phenotype is more sensitive to – or intolerant of – small differences in *Rasgrf1* expression level is because it IS sensitive enough to respond to changes in expression level, in a way that is plastic and flexible throughout life, in contrast to the growth phenotype, where parameters are set

early and are unresponsive to later changes in *Rasgrf1* expression. Mechanistically, this flexibility could be due to neuronal differences in the brain structures responsible for the production of each phenotype – neuronal remodeling, reorganization, and neurogenesis occur in the hippocampus and olfactory system in response to experience, but similar mechanisms may not facilitate hypothalamic restructuring with maturity. Thus, once the initial periods of neuronal development and pruning end, responses within the hypothalamus might be set.

Additionally, the discrepancy in sensitivity between the growth and learning phenotypes – as it relates to wild-type and *tm2*-derived expression – could be due to instances of variability in *tm2*-derived expression, other than expression level, that we have not been able to rule out. We cannot identify whether the *tm2* allele is faithfully recapitulating wild-type *Rasgrf1* expression in particular populations of cells; ie, if expression is primarily neuronal, or if glial cells are also expressing *Rasgrf1*. We see a difference in size in biallelic mice, but biallelic and wild-type mice perform similarly in the learning paradigm, meaning that *tm2*-derived expression does not augment the phenotype. This could be due to expression in sites or cells irrelevant for learning that are of importance for growth, and potentially explain why the biallelic mice present differently with respect to wild-type mice in our two studies. The timing of *tm2*-derived expression might vary as well, though this possibility seems less likely given the dependence of growth on early perturbations in expression level.

These two studies have advanced our understanding of the role *Rasgrf1* plays in the development of both of these phenotypes, but additional work could be done to make the link tighter. For example, how exactly does *Rasgrf1* influence GHRH level? Does it affect relevant transcription factors (Ikaros, Gsh-1, CREB) that we can directly assay for activation by western blotting? Does it affect the growth and maturation of GHRH-producing neurons in ways that we can examine, using neurite outgrowth assays in primary neuronal cultures from our imprinting mutants? Or does it affect an input further upstream of GHRH, the feedback loops responsible for regulating axis function, or neuronal connections between relevant structures? Can we rescue growth deficiencies in our null mice by treating them with GHRH or GH? Can we reduce overgrowth in our biallelic mice by inhibiting excess axis function? Would the effects of these treatments vary with treatment duration, or age? How exactly does *Rasgrf1* produce differences in learning and memory? Are these differences due to variations in canonical signaling (Ras, Rac) pathways? Are they olfactory learning-specific biochemical changes? Would we also see differences on a cellular level, with regard to neurite outgrowth or neuronal maturation in olfactory bulb, hippocampus, and cortex? When precisely do differences in task performance attenuate? Would other learning assays be more informative? Could we 'rescue' differences in performance by augmenting affected downstream pathways? If an antibody specific for RasGRF1 is ever easily available, a variety of immunohistochemical and biochemical approaches could be employed to identify RasGRF1 interacting partners and protein localization, both on a subcellular and structural level. Thus, there are many questions remaining to be answered, though the work presented here has narrowed the potential

focus of future inquiry and augmented our understanding of the roles *Rasgrf1* plays in learning and growth, as well as the importance of proper imprinted expression and the evolution of genomic imprinting.

APPENDICES

V.1 PANCREATIC *RASGRF1* EXPRESSION

The size phenotype we identified in our imprinting mutants (Chapter 2) could have emerged as a result of altered pancreatic function. Several studies have indicated the presence of *Rasgrf1* expression in pancreatic tissues (Guerrero *et al.*, 1996; Arava *et al.*, 1999; Font de Mora *et al.*, 2003) notably in the beta-cells (ß-cells) that comprise the endocrine pancreas, though expression has also been identified in exocrine tissues. The pancreas is a key component in pathways regulating insulin signaling and glucose homeostasis, and pancreatic islets contain the insulin-producing ß-cells, as well as glucagon-producing alpha-cells, and several other cell types. The exocrine (acinar) pancreas produces digestive enzymes that are released into the small intestine. We initially considered that the pancreas might play a role when it became apparent that the observed differences in size among our genotypes persisted beyond the point at which *Rasgrf1* becomes biallelically expressed, suggesting that perhaps expression in a tissue other than brain might be mediating the presentation of the phenotype.

Font de Mora *et al* (2003) identified a correlation between *Rasgrf1* expression and circulating insulin levels, rates of lipid catabolism, signaling via the insulin pathway, ß-cell number, and function in wild-type and *Rasgrf1* knockout mice. *Rasgrf1* null mice were found to be hypoinsulinemic, glucose intolerant, and leaner due to increased rates of lipid catabolism relative to wild-type animals. Null mice were also found to have pancreatic islets that

contained a reduced number of ß-cells, which led to reductions in overall islet size and area, as well as rates of proliferation and neogenesis. When islet protein preparations from wild-type and null mice were stimulated with IGF-1, differences in activated downstream signaling intermediates – including Akt and ERK – were identified. Activation of these intermediates in *Rasgrf1* knockout islets was significantly reduced relative to activation in wild-type islets, suggesting that *Rasgrf1* plays a role in transducing signals in the insulin pathway. We therefore hypothesized that *Rasgrf1* expression in pancreas could be a critical player in the production of size differences that we observe among biallelic, null, monoallelic maternal, and wild-type mice (Chapter 2), by affecting the functioning of those cells responsible for insulin production and signaling.

I attempted first to detect pancreatic *Rasgrf1* expression, and furthermore, to ask whether a) *Rasgrf1* is also imprinted in pancreas, and b) whether expression levels vary by genotype. We hypothesized that variations specifically in islet *Rasgrf1* expression may contribute to functional variability resulting in differences in size that correspond to the amount of *Rasgrf1* expressed in islets; this pattern would be expected if expression were imprinted in pancreas, and if the mutant *tm1* and *tm2* alleles behaved as they do in brain.

Methods used to experimentally address this hypothesis included analysis of *Rasgrf1* mRNA levels derived from whole pancreatic extracts, as well as analysis of islet-specific transcripts, both in adult and neonatal animals. Whole pancreas was dissected and homogenized from adult animals

derived from B6/PWK reciprocal crosses, and RNA was reverse transcribed into cDNA; a restriction site polymorphism distinguishes B6 from PWK-derived *Rasgrf1* transcript, so PCR analysis followed by HhaI digest allows for identification of any parent-of-origin specific expression patterns. RNA yield from whole pancreas extracts was low, as the stability of transcripts in this tissue is low.

To circumvent this problem, as well as facilitate a more specific examination of endocrine *Rasgrf1* transcript, I isolated islets from adult and neonatal animals (derived from B6/PWK reciprocal crosses) and attempted to both qualitatively and quantitatively assess islet *Rasgrf1* transcript. To do this, mice were anesthetized, and the pancreas was perfused with HBSS through the common bile duct (clamped at the liver and duodenal ends), which facilitates survival of ß-cells during the extraction process. The perfused pancreas was then removed, chopped, and digested using collagenase type IV (37C x 20min). Tissues were spun at 4C, 800 rpm, and washed to remove collagenase. Digested tissue was then resuspended in 28% Ficoll, and a Ficoll gradient was layered on top of the initial resuspension (23%, 21%, 11% Ficoll). Centrifugation in the gradient (2250rpm, 4C, 7min) facilitates the localization of islets at the interface between Ficoll layers, though that particular procedure can also be accomplished by eye – with a microscope – as the morphology of islets differs from that of acinar material.

Isolated islets were then homogenized, RNA was extracted, and cDNA was made. Despite repeated attempts to amplify *Rasgrf1* transcript from islet preparations, no transcript was reliably detected. Nested PCR assays were

developed and also failed to produce results. To verify that usable RNA was present, I simultaneously amplified a pancreas-specific transcript, *Ins2*, and that amplification was successful. *Rasgrf1* transcript did show up after some PCR amplification, but I couldn't confidently rule out contamination from brain cDNAs (used as imprinting and positive PCR controls) as the source of bands in those lanes, since amplification was sporadic, even from the same islet preparations.

Thus, despite attempts to determine whether pancreatic *Rasgrf1* expression contributes to the growth phenotype, I was unable to do so, suggesting that *Rasgrf1* is either not expressed in pancreas, is expressed – but at a very low (and potentially physiologically irrelevant) level, or is expressed, but in a form that is undetectable by our PCR primers. Additionally, the results of the size phenotype characterization (Chapter 2) are convincing in that they implicate the GH/IGF-1 axis as playing a primary role in the production of proportional differences in size, with *Rasgrf1* affecting neural inputs to the axis. IGF-1 does play a role in islet biology, and it is conceivable that pancreatic contributions to the size phenotype are present, but they are likely the result of differences in circulating IGF-1 levels that result from *Rasgrf1*-affected upstream signaling.

V.2 RNA *IN SITU* HYBRIDIZATION AND IMMUNOHISTOCHEMISTRY

Introduction

As the results of the behavioral analysis indicate, the *tm2* allele does not sufficiently recapitulate wild-type *Rasgrf1* expression (see Chapter 3). We hypothesized that this discrepancy in expression could be due to the presence of the PGK enhancer sequence inserted in place of the repeats that are normally 3′ of the DMD (**Figure V.2.1**).

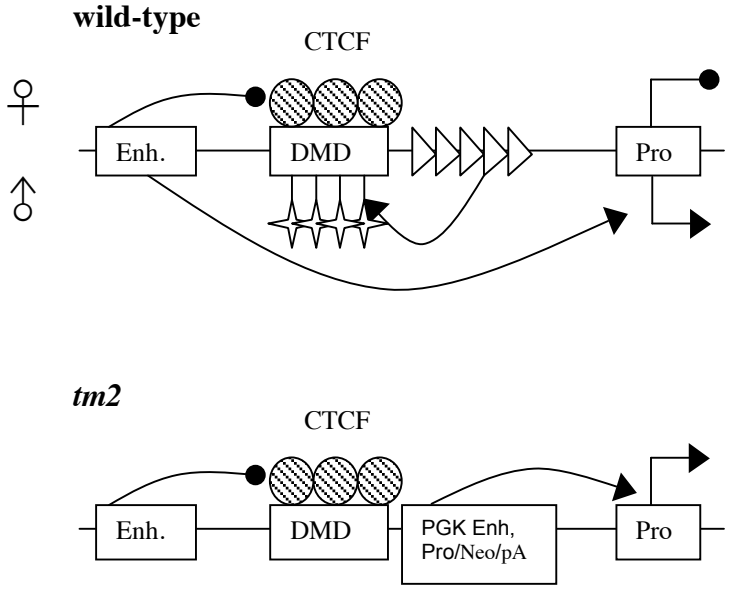


FIGURE V.2.1. Schematic of the *Rasgrf1* wild-type and *tm2* alleles, depicting the insertion of the PGK promoter/enhancer/pA and neo sequences in place of the repeats.

As PGK is a 'housekeeping' gene, we considered that its enhancer may create different expression patterns throughout the brain, either with respect

to cell type or structural distribution, in addition to altering expression level relative to wild-type allele activity. Thus, we attempted to use RNA *in situ* hybridization to localize *Rasgrf1* transcript within brain, with expression derived from the wild-type and *tm2* alleles, and to visualize cellular RasGRF1 localization using double-labeled immunohistochemical detection. We hoped that *in situ* hybridization would provide visible patterns of *Rasgrf1* expression on a more macrostructural level and identify any allele-specific differences in transcript distribution. We hoped that immunohistochemical staining would help to identify where RasGRF1 protein is present, on a more microstructural (subcellular) level, and that double-labeling with neuronal markers would facilitate identification of any allele-specific differences in cell type.

Materials and Methods

In Situ Hybridization

P8 brains from wild-type, +/*tm2*, and null animals were dissected, embedded, and frozen in Tissue-Tek O.C.T.© compound. Blocks were then mounted and crysectioned into 15uM slices, which were mounted 6/slide. Brains were sectioned horizontally and coronally.

After sectioning, slides were fixed in 4% paraformaldehyde dissolved in 0.1M PBS for 12min at room temperature, followed by two 1X PBS washes (5min each). Sections were then acetylated in triethanolamine-acetic anhydride for 10 min (room temperature), washed twice in 1X PBS (5min), and blocked at room temperature in hybridization buffer (0.1% Tween, 50%

formamide, 5X SSC, 5X Denhardt's, 5mM EDTA, 10mM NaH_2PO_4 pH 8.0, 50mM Tris pH 8.0, 250ug/ml salmon sperm DNA, 100ug/ml yeast RNA) for two hours. Slides were probed overnight at 55C, in a box containing 50% formamide/3X SSC. The next day, slides were washed twice in 5X SSC (65C X 15min), twice in 0.2X SSC (65C x 15min), once in 0.2X SSC (room temperature x 15min), once in 1X B1 buffer (0.1M Tris, 0.15M NaCl; room temperature x 10min), and blocked with heat-inactivated goat serum (in B1 buffer) for 1 hour at room temperature. Slides were then probed with an alkaline-phosphatase-conjugated anti-digoxigenin secondary antibody (1:3000) at 4C overnight. On day three, slides were washed three times with 1X B1 buffer (room temperature x 5 minutes), twice in B3 buffer (0.1M Tris, 0.1M NaCl, 0.05M $MgCl_2$; room temperature x 5min), and stained using NBT-BCIP (in B3) buffer. Slides were stained until color (purple) began to develop, then washed twice in 1X PBS for 10min, and fixed at 4C for at least six hours. Sense controls were run simultaneously with antisense probes, on slides containing parallel sections, and developed for the same length of time as antisense probes. On day four, slides were washed in 1X PBS (room temperature x 10minutes), coverslipped, and sealed. All reagents and glassware were RNAse free.

Probe Synthesis

Riboprobes were synthesized and targeted the portion of the *Rasgrf1* transcript that does not share sequence identity with *Rasgrf2*. Gene sequences were BLASTed, and non-overlapping portions were identified. Three probes were designed, of varying lengths, covering two non-homologous regions,

and sequences were cloned into pGEM TEasy vectors using T4 ligase. Colonies were miniprepped and digested to indicate directionality of the insert. Remaining plasmids were then linearized, and T7 polymerase was used to synthesize the riboprobes in a transcription reaction employing rNTPs and digoxigenin-labeled UTP, which acts as a substrate for the anti-digoxigenin secondary antibody. Sense and antisense transcription reactions were carried out for each probe, and sense probes acted as a negative control during *in situ* hybridization. Two probes were selected for further use, termed "1A" and "1/2". Primer sequences used to amplify probe 1A: (F) 5'-GTG GTC CTG GAC AAG CTG AT-3'; (R) 5'-GTG TTG GCC AAA GAC ATC CT-3'. Primer sequences used to amplify probe "1/2": (F) 5'-CTG TAC GGA GAT GCC CCT AA-3'; (R) 5'-TCG AAG GGC TCA GTC TTC AC-3'.

Immunohistochemistry

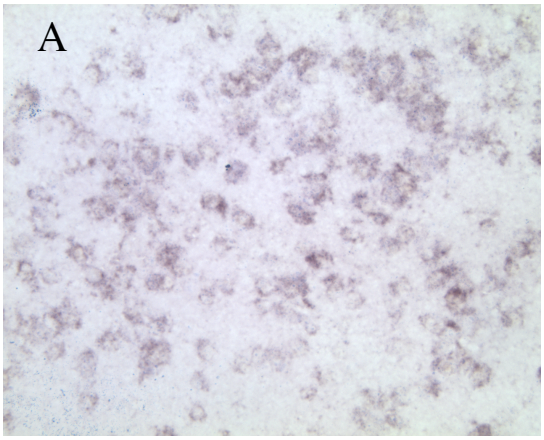
For IHC analysis, brains from biallelic and null P8 animals (to use as controls) were formalin-fixed, paraffin-embedded, and sectioned using the College of Veterinary Medicine's histology lab. Two sections were placed on a slide, so that positive and negative controls could be run simultaneously. Slides were then processed by Patricia Fisher (in the Immunopathology Research and Development Laboratory) using anti-RasGRF1 antibody (sc-224; Santa Cruz Biotechnology[SCBT]) anti-neuron-specific enolase (NSE, which specifically labels neuronal cytoplasm; DAKO A587), and anti-MAP2 (microtubule-associated protein, which labels dendrites; Chemicon AB5622). Fluorescent-conjugated secondaries were used to facilitate co-localization of proteins.

Results and Discussion

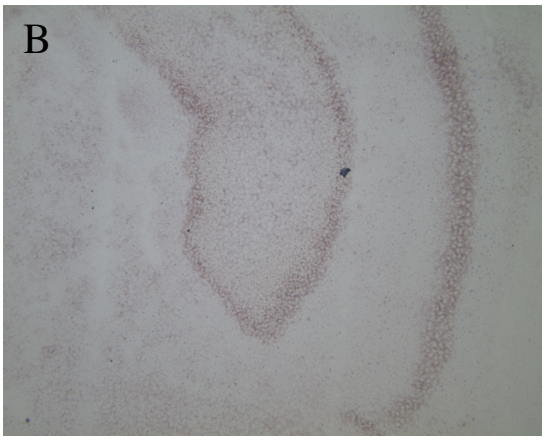
In Situ Hybridization

Wild-type brains (**Figure V.2.2, a-d**) stained well, with clearly distinct populations of *Rasgrf1*-expressing cells. Sense controls displayed no staining (**Figure 2e**). +/*tm2* brains also produced clear patterns of *Rasgrf1* staining with antisense probes (**Figure V.2.3a-d**). Sense probes produced no staining (**Figure V.2.3e**).

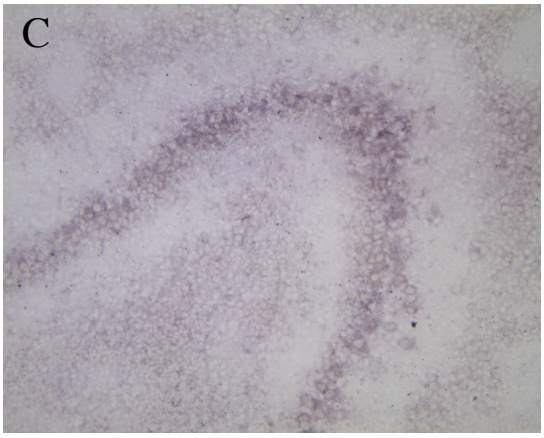
In addition to staining wild-type and +/*tm2* brains, I also stained P8 null brains from animals that had been verified to be unmethylated at the *tm1* DMD. These brains produced staining patterns very similar to those observed in the wild-type and +/*tm2* animals (**Figure V.2.4**), suggesting that despite my attempts to generate a *Rasgrf1*-specific probe, I was working with a probe that identified non-*Rasgrf1* transcripts. I re-aligned my probe sequences against the mouse genome and retrieved the same results I had earlier – by my best estimation, the sequences I was targeting were unique to *Rasgrf1*. At this point, we decided that more convincing results might be obtained by pursuing transcript quantification within brain structures, and the focus of this work shifted toward that.



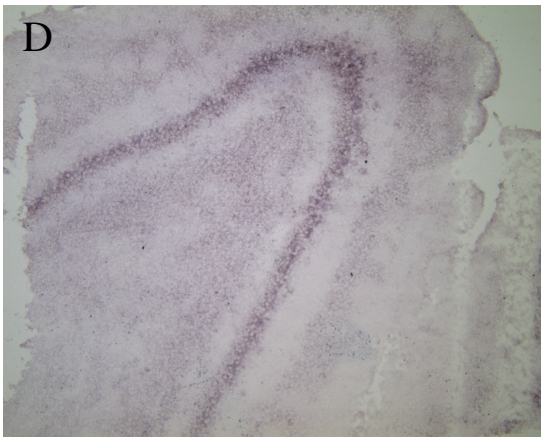
Wild-type cerebellum, probe 1A



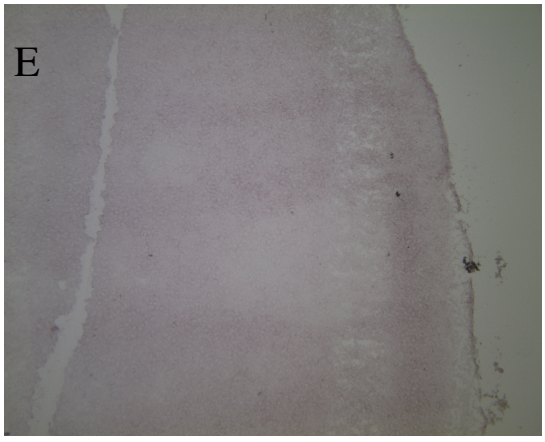
Wild-type hippocampus, probe 1A



Wild-type olfactory bulb, probe 1/2

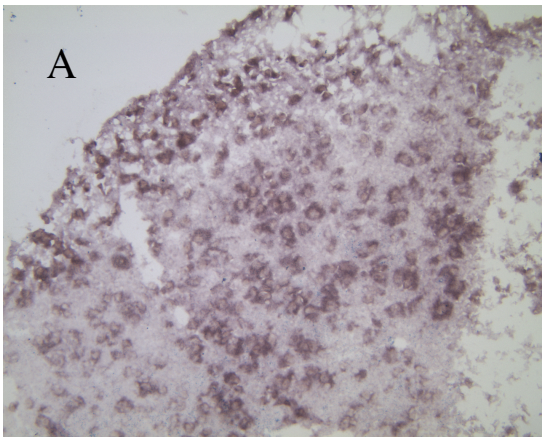


Wild-type olfactory bulb, probe 1/2

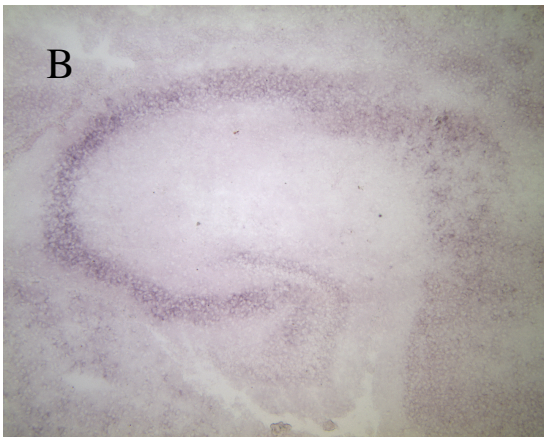


Wild-type, sense probe

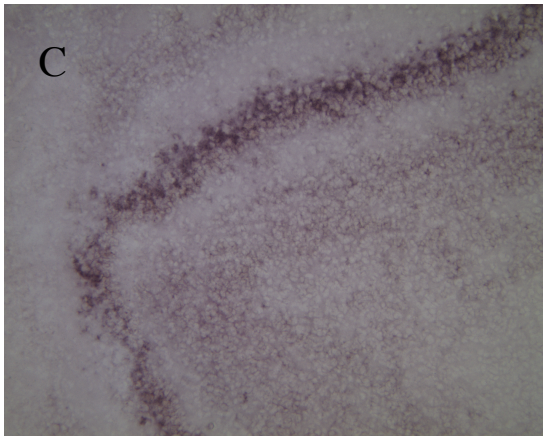
FIGURE V.2.2. *In situ* hybridization images using probes for *Rasgrf1* in P8 wild-type brains. Brains were sectioned either coronally (a, b) or horizontally (c, d) and probed with dig-labeled antisense riboprobes (a-d). Sense probes (e) were used as controls for nonspecific staining.



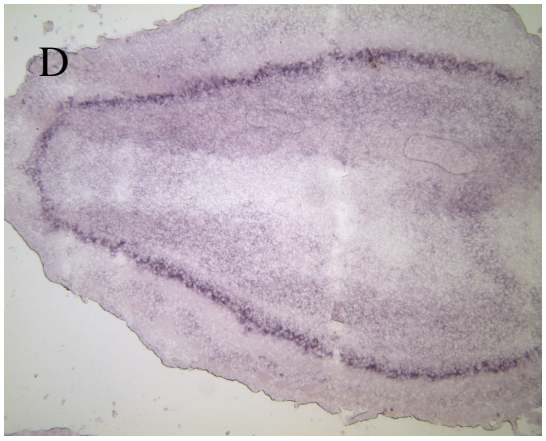
+/*tm2* cerebellum, probe 1A



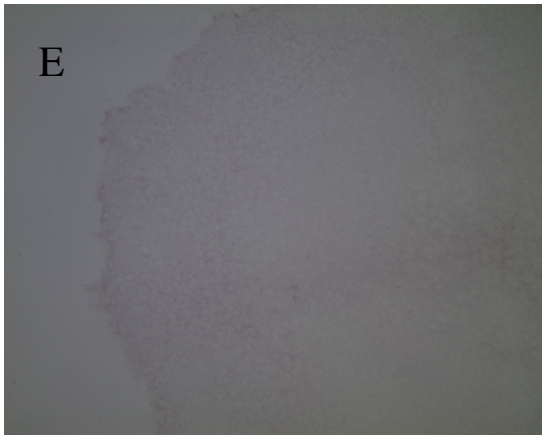
+/*tm2* hippocampus, probe 1/2



+/*tm2* olfactory bulb, probe 1/2

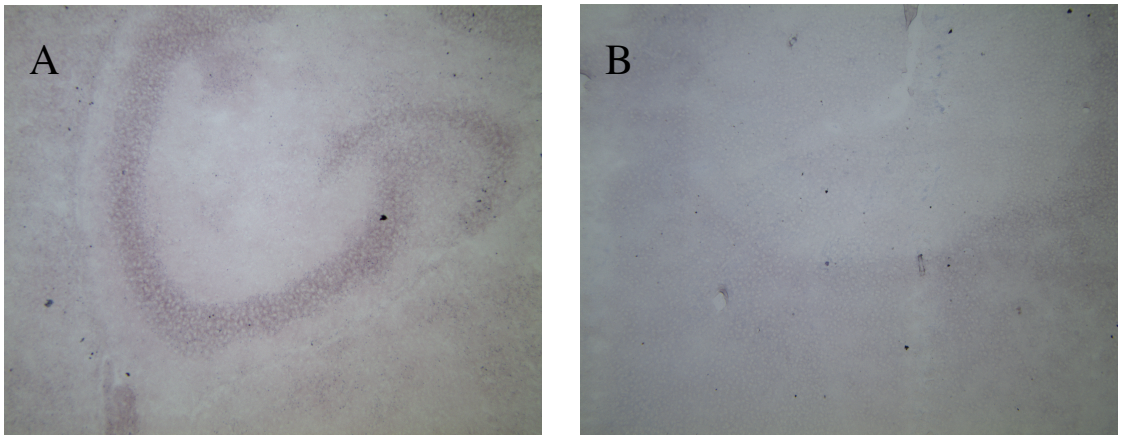


+/*tm2* olfactory bulb, probe 1/2



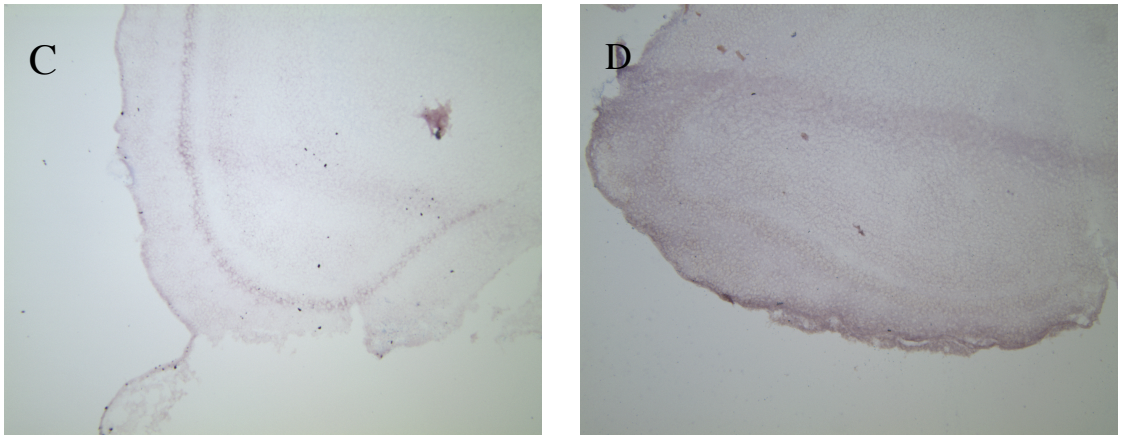
+/*tm2* olfactory bulb, sense probe

FIGURE V.2.3. *In situ* hybridization of images using probes for *Rasgrf1* in P8 +/*tm2* brains. Brains were sectioned either coronally (a, b) or horizontally (c, d) and probed with dig-labeled antisense riboprobes (a-d). Sense probes (e) were used as controls for nonspecific staining.



Null hippocampus, probe 1A

Null hippocampus, sense probe



Null olfactory bulb, probe 1A

Null olfactory bulb, sense probe

FIGURE V.2.4. *In situ* hybridization of images using probes for *Rasgrf1* in P8 null brains. Brains were sectioned horizontally and probed with dig-labeled antisense riboprobes (a, c). Sense probes (b, d) were used as controls for nonspecific staining, and demonstrate that the staining seen in null brains is indeed specific.

Should one attempt to repeat *in situ* hybridization in the future, improvements on the protocol could be made by re-synthesizing probes (as the ones I made will be several years old at that point), increasing the stringency of the hybridization and wash conditions, and RNAse-treating sections post-hybridization to remove any background probe. Alternatively, one might wish to switch to fluorescent *in situ* hybridization, where there is no colorimetric reaction step, which removes some of the subjectivity in detection.

Immunohistochemistry

Brains from biallelic and null mice were processed with antibodies allowing for colocalization of RasGRF1 with neuronal markers, using fluorescence-conjugated secondaries. Initially, IHC protocols worked very well, producing images suggesting that RasGRF1 is present in neuronal cytoplasm (**Figure V.2.5**). However, when null brains were processed as negative controls, RasGRF1 signal was again identified (**Figure V.2.6**), similar to the result we had obtained when processing the *in situ* hybridizations. The addition of blocking peptide to the protocol indicated that the signals we were seeing were in fact due to antibody-antigen recognition, and we not the result of nonspecific antibody activity (**figure V.2.6**), suggesting either that the supplied RasGRF1 antibody was not RasGRF1-specific, or that our null brains were not actually null.

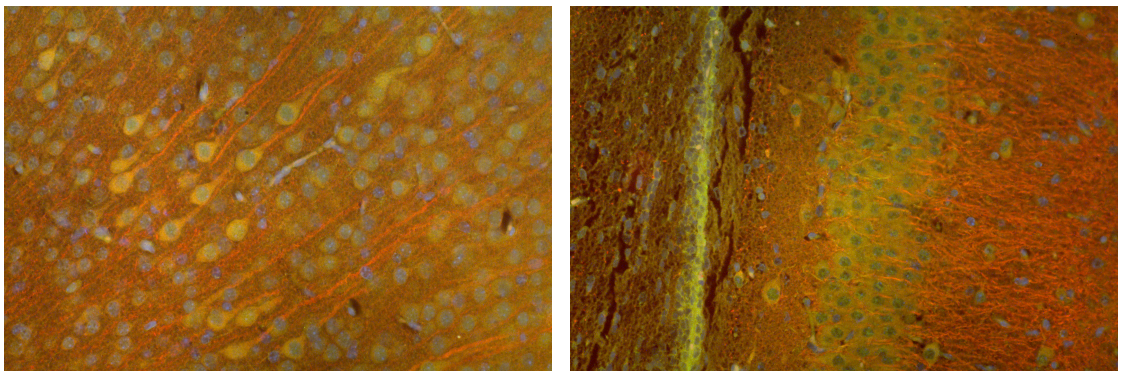


FIGURE V.2.5. IHC images produced by double-labeling P8 biallelic hippocampal coronal sections with RasGRF1 and NSE antibodies. RasGRF1 is labeled in green, and NSE is in red. Colocalization of the two antibodies is present in the orange areas.

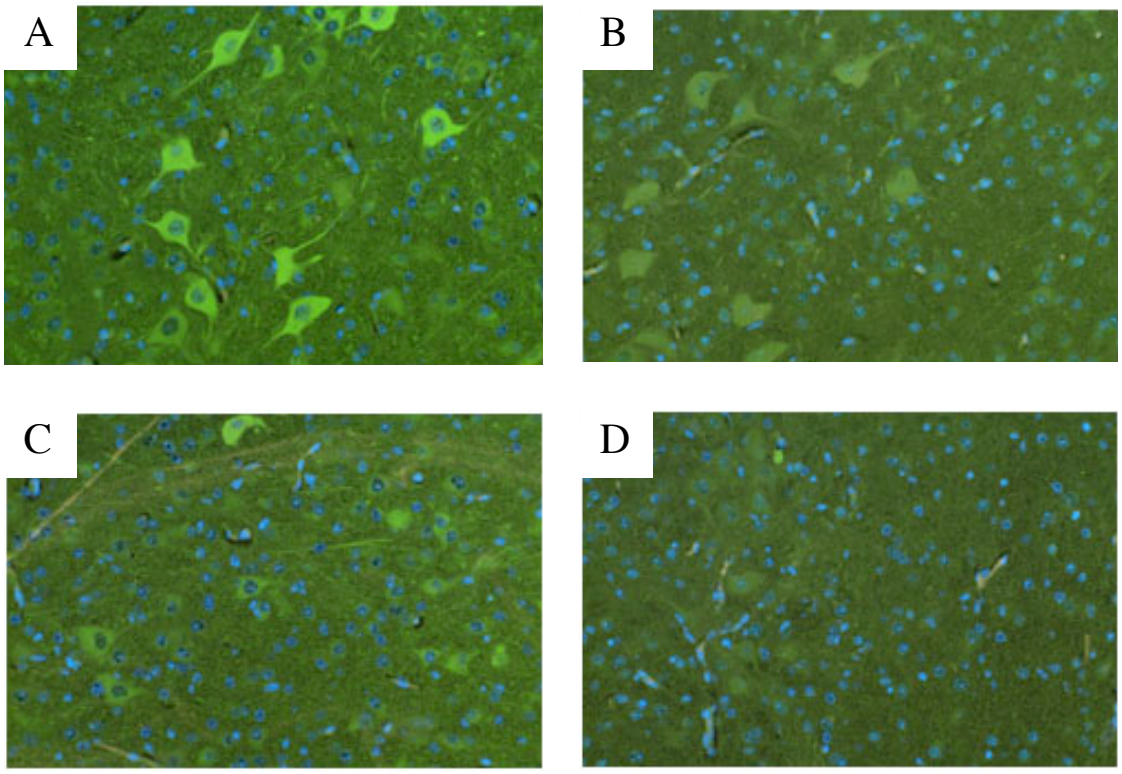


FIGURE V.2.6. Biallelic (a, b) and null (c, d) coronal sections probed with RasGRF1 antibody (green). Sections in (b) and (d) were treated with blocking peptide, which removed the fluorescence associated with RasGRF1 antibody recognition.

I verified that the brains submitted were unmethylated, thus no RasGRF1 should be present at the age assayed. Upon more extensive review of the antibody purchased, we concluded that it cross-reacted to a degree with RasGRF2 (**FIGURE V.2.7**), which is the likely source for the presence of antibody signal in the null brains.

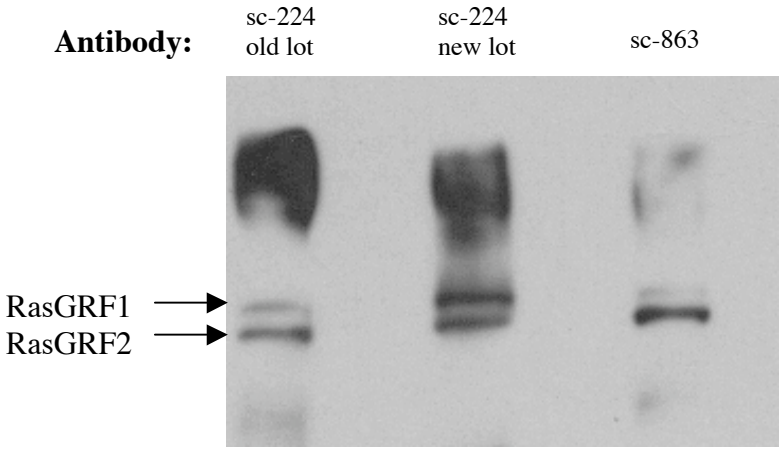


FIGURE V.2.7. Western blots demonstrating cross-reactivity of RasGRF1 antibodies with RasGRF2. Three different antibodies were tested: sc-224 (original lot), sc-224 (new lot), and a third antibody from SCBT, sc-863. All antibodies produced cross-reactivity. Brains from wild-type animals were used for protein preparations. (figure, Krista Kauppinen)

As part of this process, we reviewed the citations provided by Santa Cruz Biotechnology for the antibody purchased (RasGRF1 C-20, sc-224) :Tian & Feig, 2006, reported cross-reaction of the same antibody with other RasGRF family members; Baldassa *et al.*, 2007, reported cross reaction of the same antibody with RasGRF2 (see figure 2 in paper); Forlani *et al.*, 2006, reported cross reaction with RasGRF2 (see figure 1 in paper); Tian *et al.*, 2004, reported cross reaction with RasGRF2 (see figure 1 in paper). Essentially, the only studies not reporting cross-reactivity were those in which RasGRF1 was transfected into cell lines, where RasGRF2 might not be present endogenously. As such, this particular approach to studying the subcellular distribution of wild-type and *tm2*-derived RasGRF1 protein will be difficult until a truly specific primary antibody is available.

REFERENCES

Alba M, Salvatori R (2004) A mouse with target ablation of the growth hormone-releasing hormone gene: a new model of isolated growth hormone deficiency. Endocrinology **145**, 4134-43.

Allen Brain Atlas, http://www.brain-map.org

Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Transgenerational actions of endocrine disruptors and male fertility. Science **308**, 1466-9.

Arava Y, Seger R, Walker MD (1999) GRFbeta, a novel regulator of calcium signaling, is expressed in pancreatic beta cells and brain. Journal of Biological Chemistry **274**, 24449-52.

Arozarena I, Matallanas D, Berciano MT, Sanz-Moreno V, Calvo F, Munoz MT, Egea G, Lafarga M, Crespo P (2004) Activation of H-Ras in the endoplasmic reticulum by the RasGRF family guanine nucleotide exchange factors. Molecular and Cellular Biology **24**, 1516-30.

Baldassa S, Gnesutta N, Fascio U, Sturani E, Zippel R (2007) SCLIP, a microtubule-destabilizing factor, interacts with RasGRF1 and inhibits its ability to promote Rac activation and neurite outgrowth. Journal of Biological Chemistry **282**, 2333-45.

Barker DJ (1990) The fetal and infant origins of adult disease. BMJI **301**, 1111.

Barlow DP, Stoger R, Herrmann BG, Saito K, Schweifer N (1991) The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. Nature **349**, 84-7.

Barreto G, Schafer A, Marhold J, Stach D, Swaminathan SK, Handa V, Doderlein G, Maltry N, Wu W, Lyko F, Niehrs C (2007) Gadd45a promotes epigenetic gene activation by repair-mediated DNA methylation. Nature **445**, 671-675.

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K (2007) High-resolution profiling of histone methylations in the human genome. Cell **129**, 823-37.

Beaudet AL & Jiang Y (2002) A rheostat model for a rapid and reversible form of imprinting-dependent evolution. American Journal of Human Genetics **70**, 1389-1397.

Bell AC and Felsenfeld G (2000) Methylation of the CTCF-dependent boundary control imprinted expression of the Igf2 gene. Nature **405**, 482-5.

Bell AC, West AG, Felsenfeld G (1999) The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. Cell **98**, 387-96.

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell **125**, 315-26.

Bird A (2002) DNA methylation patterns and epigenetic memory. Genes and Development **16**, 6-21.

Boguski MS and McCormick F (1993) Proteins regulating Ras and its relatives. Nature **366**, 643-54.

Boyes J and Bird A (1991) DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell **64**, 1123-34.

Brambilla R, Gnesutta N, Minichiello L, White G, Roylance AJ, Herron CE, Ramsey M, Wolfer DP, Cestari V, Rossi-Arnaud C, Grant SGN, Chapman PF, Lipp H-P, Sturani E, and Klein R (1997) A role for the Ras signaling pathway in synaptic transmission and long-term memory. Nature **390**, 281-286.

Brideau CM, Eilertson KE, Bustamante CD, Soloway PD (2009) Successful computation prediction of novel imprinted genes. Submitted.

Buchsbaum R, Telliez JB, Goonesekera S, Feig LA (1996) The N-terminal pleckstrin, coiled-coil, and IQ domains of the exchange factor Ras-GRF act cooperative to facilitate activation by calcium. Molecular and Cellular Biology **16**, 4888-96.

Bull JH and Wootton JC (1984) Heavily methylated amplified DNA in transformants of Neurospora crassa. Nature **310**, 701-4.

Cen H, Papageorge AG, Vass WC, Zhang KE, Lowy DR (1993) Regulated and constitutive activity of CDC25mM (GRF), a Ras-specific exchange factor. Molecular and Cellular Biology **13**, 7718-24.

Chandler VL (2007) Paramutation: from maize to mice. Cell **128**, 641-5.

Clapcott SJ, Peters J, Orban PC, Brambilla R, Graham CF (2003) Two ENU-induced mutations in Rasgrf1 and early mouse growth retardation. Mammalian Genome **14**, 495-505.

Cleard F, Delattre M, Spierer P (1997) SU(VAR)3-7, a Drosophila heterochromatin-associated protein and companion of HP1 in the genomic silencing of position-effect variegation. The EMBO Journal **16**, 5280-8.

Cox GF, Burger J, Lip V, Mau UA, Sperling K, Wu BL, Horsthemke B (2002) Intracytoplasmic sperm injection may increase risk of imprinting defects. American Journal of Human Genetics **71**, 162-164.

de la Puente A, Hall J, Wu YZ, Leone G, Peters J, Yoon BJ, Soloway P, Plass C (2002) Structural characterization of Rasgrf1 and a novel linked imprinted locus. Gene **291**, 287-97.

DeChiara TM, Efstratiadis A, Robertson EJ (1990) A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature **345**, 78-80.

Delaval K and Feil R (2004) Epigenetic regulation of mammalian genomic imprinting. Current Opinions in Genetics and Development **14**, 188-95.

Delaval K, Govin J, Cerqueira F, Rousseax S, Khochbin S, Feil R (2007) Differential histone modifications mark mouse imprinting control regions during spermatogenesis. The EMBO Journal **26**, 720-9.

Dobosy JR and Selker EU (2001) Emerging connections between DNA methylation and histone acetylation. Cellular and Molecular Life Sciences **58**, 721-7.

Dobrowolny G, Giacinti C, Pelosi L, Nicoletti C, Winn N, Barberi L, Molinaro M, Rosenthal N, Musaro A (2005) Muscle expression of a local Igf-1 isoform protects motor neurons in an ALS mouse model. Journal of Biological Chemistry **168**, 19309.

Drake NM, Park YJ, Shirali AS, Cleland TA, Soloway PD (2009) Imprint switch mutations at Rasgrf1 support conflict hypothesis of imprinting and define a growth control mechanism upstream of IGF1. Mammalian Genome, epub June 10.

Edwards CA and Ferguson-Smith AC (2007) Mechanisms regulating imprinted genes in cluster. Current Opinions in Cell Biology **19**, 281-9.

Edwards CA, Rens W, Clarke O, Mungall AJ, Hore T, Graves JA, Dunham I, Ferguson-Smith AC, Ferguson-Smith MA (2007) The evolution of imprinting: chromosomal mapping of orthologues of mammalian imprinted domains in monotreme and marsupial mammals. BMC Evolutionary Biology **7**,157.

Elango N, Hunt BG, Goodisman MA, Yi SV (2009) DNA methylation is widespread and associated with differential gene expression in castes of the honeybee, Apis mellifera. Proceedings of the National Academy of Sciences **106**, 11206-11.

Ezzat S, Mader R, Fischer S, Yu S, Ackerley C, Asa SL (2006) An essential role for the hematopoietic transcription factor Ikaros in hypothalamic-pituitary-mediated somatic growth. PNAS **103**, 2214-9.

Ezzat S, Yu S, Asa SL (2005) The zinc finger Ikaros transcription factor regulates pituitary growth hormone and prolactin gene expression through distinct effects on chromatin accessibility. Molecular Endocrinolology **19**, 1004-11.

Fam NP, Fan WT, Wang Z, Zhang LJ, Chen H, Moran MF (1997) Cloning and characterization of Ras-GRF2, a novel guanine nucleotide exchange factor for Ras. Molecular and Cellular Biology **17**, 1396-406.

Fan WT, Koch CA, de Hoog CL, Fam NP, Moran MF (1998) The exchange factor Ras-GRF2 activates Ras-dependent and Rac-dependent mitogen-activated protein kinase pathways. Current Biology **8**, 935-8.

Farnsworth CL, Freshney NW, Rosen LB, Ghosh A, Greenberg ME, Feig LA (1995) Calcium activiation of Ras mediated by neuronal exchange factor Ras-GRF. Nature **376**, 524-527.

Feinberg AP and Vogelstein B (1983) Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature **301**, 89-92.

Ferguson-Smith AC, Sasaki H, Cattanach BM, Surani MA (1993) Parental-origin-specific epigenetic modification of the mouse H19 gene. Nature **362**, 751-5.

Fernandez-Medarde A, Esteban LM, Nunez A, Porteros A, Tessarollo L, Santos E (2002) Targeted disruption of Ras-GRF2 shows its dispensability for mouse growth and development. Molecular and Cellular Biology **22**, 2498-504.

Fernandez-Medarde A, Porteros A, de la Rivas J, Nunez A, Fuster JJ, Santos E (2007) Laser microdissection and microarray analysis of the hippocampus of Ras-GRF1 knockout mice reveals gene expression changes affecting signal transduction pathways related to memory and learning. Neuroscience **146**, 272-85.

Ferrari C, Zippel R, Martegani E, Gnesutta N, Carrera V, Sturani E (1994) Expression of two different products of CDC25Mm, a mammalian Ras activator, during development of mouse brain. Exp Cell Res **210**, 353-357.

Filson AJ, Louvi A, Efstratiadis A, and Robertson EJ (1993) Rescue of the T-associated maternal effect in mice carrying null mutations in Igf-2 and Igf2r, two reciprocally imprinted genes. Development **118**, 731-736.

Font De Mora J, Estaban LM, Burks DJ, Nunez A, Garces C, Garcia-Barrado MJ, Iglesias-Osma MC, Moratinos J, Ward JM, and Santos E(2003) Ras-GRF1 signaling is required for normal B-cell development and glucose homeostasis. The EMBO Journal **22**, 3039-3049.

Forlani G, Baldassa S, Lavagni P, Sturani E, Zippel R (2006) The guanine nucleotide exchange factor RasGRF1 directly binds microtubules via DhPH2-mediated interaction. FEBS J **273**, 2127-38.

Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu YZ, Plass C, Esteller M (2005) Epigenetic differences arise during the lifetime of monozygotic twins. PNAS **102**, 10604-9.

Gehring M, Huh JH, Hsieh TF, Penterman J, Choi Y, Harada JJ, Goldberg RB, Fischer RL (2006) DEMETER DNA glycosylase establishes MEDEA polycomb gene self-imprinting by allele-specific demethylation. Cell **124**, 495-506.

Giese KP, Friedman E, Telliez J, Fedorov NB, Wines M, Feig LA, and Silva AJ (2001) Hippocampus-dependent learning and memory is impaired in mice lacking the Ras-guanine-nucleotide releasing factor 1 (Ras-Grf1). Neuropharmacology **41**, 791-800.

Goll MG, Bestor TH (2005) Eukaryotic cytosine methyltransferases. Annual Review of Biochemistry **74**, 481-514.

Gong Z, Morales-Ruiz T, Ariza RR, Roldan-Arjona T, David L, Zhu JK (2006) ROS1, a repressor of transcriptional gene silencing in Arabidopsis, encodes a DNA glycosylase /lyase. Cell **111**, 803-14.

Gotoh T, Niino Y, Tokuda M, Hatase O, Nakamura S, Matsuda M, Hattori S (1997) Activation of R-Ras by Ras-guanine nucleotide-releasing factor. Journal of Biological Chemistry **272**, 18602-7.

Graff J, Mansuy IM (2009) Epigenetic dysregulation in cognitive disorders. European Journal of Neuroscience **30**, 1-8.

Grewal SI, Klar AJ (1996) Chromosomal inheritance of epigenetic states in fission yeast during mitosis and meiosis. Cell **86**, 95-101.

Guerrero C, Rojas JM, Chedid M, Esteban LM, Zimonjic DB, Popescu NC, Font de Mora J, Santos E (1996) Expression of alternative forms of Ras exchange factors GRF and SOS1 in different human tissues and cell lines. Oncogene **12**, 1097-107.

Hegedus B, Yeh TH, Lee da Y, Emnett RJ, Li J, Gutmann DH (2008) Neurofibromin regulates somatic growth through the hypothalamic-pituitary axis. Human Molecular Genetics **17**, 2956-66.

Hikichi T, Kohda T, Kaneko-Ishino T, Ishino F (2003) Imprinting regulation of the murine Meg1/Grb10 and human GRB10 genes; roles of brain-specific promoters and mouse-specific CTCF binding sites. Nucleic Acids Research **31**, 1398-406.

Hosui A and Hennighausen L (2008) Genomic dissection of the cytokine-controlled Stat5 signaling network in liver. Physiological Genomics **34**, 135-143.

Hu JF, Oruganti H, Vu TH, Hoffman AR (1998) Tissue-specific imprinting of the mouse insulin-like growth factor II receptor gene correlates with differential allele-specific DNA methylation. Molecular Endocrinology **12**, 220-32.

Innocenti M, Zippel R, Brambilla R, Sturani E (1999) CDC25Mm/Ras-GRF1 regulates both Ras and Rac signaling pathways. FEBS Letters **460**, 357-362.

Itier J-M, Tremp GL, Leonard J-F, Multon M-C, Ret G, Schweighoffer F, Tocque B (1998) Imprinted gene in postnatal growth role. Nature **393**,125-126.

Jablonka E, Raz G (2009) Transgenerational epigenetic inheritance: prevalence, mechanisms, implications for the study of heredity and evolution. The Quarterly Review of Biology **84**, 131-76.

Jaenisch R and Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nature Genetics **33**, Suppl: 245-54.

Jenuwein T and Allis CD (2001) Translating the histone code. Science **293**, 1074-80.

Kaati G, Bygren LO, Edvinsson S (2002) Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. European Journal of Human Genetics **10**, 682-8.

Kiyono M, Kaziro Y, Satoh T (2000) Induction of rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25(Mm) following phosphorylation by the nonreceptor tyrosine kinase Src. Journal of Biological Chemistry **275**, 5441-6.

Kiyono M, Satoh T, Kaziro Y (1999) G protein beta gamma subunit-dependent Rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25(Mm). PNAS **96**, 4826-31.

Knoepfler PS and Eisenman RN (1999) Sin meets NuRD and other tails of repression. Cell **99**, 447-450.

Krapivinsky G, Krapivinsky L, Manasian Y, Ivanov A, Tyzio R, Pellegrino C, Ben-Ari Y, Clapham DE, and Medina I (2003) The NMDA Receptor is Couple to the ERK Pathway by a Direct Interaction with NR2B and RasGRF1. Neuron **40**, 775-784.

Kronauer DJ (2008) Genomic imprinting and kinship in the social Hymenoptera: what are the predictions? Journal of Theoretical Biology **254**, 737-40.

Lavagni P, Indrigo M, Colombo G, Martegani E, Rosenblum K, Gnesutta N, Zippel R (2009) Identification of novel RasGRF1 interacting partners by large-scale proteomic analysis. Journal of Molecular Neuroscience **37**, 212-24.

Li B, Carey M, and Workman JL (2007) The role of chromatin during transcription. Cell **128**, 707-719.

Li E, Beard C, Forster AC, Bestor TH, Jaenisch R (1993) DNA methylation, genomic imprinting, and mammalian development. Cold Spring Harbor Symposia on quantitative biology **58**, 297-305.

Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell **69**, 915-26.

Li S, Tian X, Hartley DM, Feig LA (2006) Distinct roles of Ras-guanine nucleotide-releasing factor 1 (Ras-GRF1) and Ras-GRF2 in the induction of long-term potentiation and long-term depression. Journal of Neuroscience **26**, 1721-9.

Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC (2005) Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. Journal of Nutrition **135**, 1382-6.

Lillycrop KA, Phillips ES, Torrens C, Hanson MA, Jackson AA, Burdge GC (2008) Feeding pregnant rats a protein-restricted diet persistently alters the methylation of specific cytosines in the hepatic PPAR alpha promoter of the offspring. British Journal of Nutrition **100**, 278-82.

Lindroth AM, Park YJ, McLean CM, Dokshin GA, Persson JM, Herman H, Pasini D, Miro X, Donohoe ME, Lee JT, Helin K, Soloway PD (2008) Antagonism between DNA and H3K27 methylation at the imprinted Rasgrf1 locus. PloS Genetics **4**, e1000145.

Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature **462**, 315-22.

Liu JL, Grinberg A, Westphal H, Sauer B, Accili D, Karas M, LeRoith D (1998) Insulin-like growth factor-1 affects perinatal lethality and postnatal development in a gene dosage-dependent manner: manipulation using the Cre/loxP system in transgenic mice. Molecular Endocrinology **12**, 1452-62.

Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A (1993) Mice carrying null mutations of the genes encoding insulin-like growth factor 1 (Igf-1) and type 1 IGF receptor (Igf1r). Cell **75**, 59-72.

Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A (1993) Mice carrying null mutations of the genes encoding insulin-like growth factor 1 (Igf-1) and type 1 IGF receptor (Igf1r). Cell **75**, 59-72.

Ludwig T, Eggenschwiler J, Fisher P, D'Ercole AJ, Davenport ML, Efstratiadis A (1996) Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. Developmental Biology **177**, 517-35.

Luedi PP, Dietrich FS, Weidman JR, Bosko JM, Jirtle RL, Hartemink AJ (2007) Computational and experimental identification of novel human imprinted genes. Genome Research **17**, 1723-30.

Lupu F, Terwilliger JD, Lee K, Segre GV, and Efstratiadis A (2001) Roles of Growth Hormone and Insulin-Like Growth Factor 1 in Mouse Postnatal Growth. Developmental Biology **229**, 141-162.

Ma DK, Jang M-H, Guo JU, Kitabatake Y, Chang M-l, Pow-anpongkul N, Flavell RA, Lu B, Ming G-l, Song H (2009) Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science **323**, 1074-77.

Macdonald JI, Verdi JM, Meakin SO (1999) Activity-dependent interaction of the intracellular domain of the rat trkA with intermediate filament proteins, the beta-6 proteasomal subunit, Ras-GRF1, and the p162 subunit of eIF3. Journal of Molecular Neuroscience **13**, 141-58.

Mager J and Bartolomei MS (2005) Strategies for dissecting epigenetic mechanisms in the mouse. Nature Genetics **37**, 1194-200.

Maher ER, Brueton LA, Bowdin SC, Luhari A, Cooper W, Cole TR, Macdonald F, Sampson JR, Barratt CL, Reik W, Hawkins MM (2003) Beckwith-Wiedemann syndrome and assisted reproduction technology (ART). Journal of Medical Genetics **40**, 62-64.

Mancini-Dinardo D, Steele SJ, Levorse JM, Ingram RS, Tilghman SM (2006) Elongation of the Kcnq1ot1 transcript is required for genomic imprinting of neighboring genes. Genes and Devleopment **20**, 1268-82.

Martienssen RA, Kloc A, Slotkin RK, Tanurdzic M (2008) Epigenetic inheritance and reprogramming in plants and fission yeast. Cold Spring Harbor Synposia on Quantiative Biology **73**, 265-71.

Mattingly RR and Macara IG (1996) Phosphorylation-dependent activation of the Ras-GRF/CDC25Mm exchange factor by muscarinic receptors and G-protein beta gamma subunits. Nature **382**, 268-72.

Mattingly RR, Saini V, and Macara IG (1999) Activation of the Ras-GRF/CDC25mM exchange factor by lysophosphatidic acid. Cell Signaling **11**, 603-10.

Meyer CWE, Korthaus D, Jagla W, Cornali E, Grosse J, Fuchs H, Klingenspor M, Roemheld S, Tschop M, Heldmaier G, de Angelis MF, Nehls M (2004) A novel missense mutation in the mouse growth hormone gene causes semidominant dwarfism, hyperghrelinemia, and obesity. Endocrinology **145**, 2531-2541.

Mikkelsen TS, Ku M, Jaffe DB, Isaac B, Liberman E, Giannoukos G, Alvarez P, Brockman W, Kim TK, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C, Lander ES, Bernstein BE (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature **448**, 553-60.

Molnar A and Georgopoulos K (1994) The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. Molecular and Cellular Biology **14**, 8292-8303.

Molnar A, Wu P, Largespada DA, Vortkamp A, Scherer S, Copeland NH, Jenkins NA, Bruns G, Georgopoulos K (1996) The Ikaros gene encodes a family of lymphocyte-restricted zinc finger DNA binding proteins, highly conserved in human and mouse. Journal of Immunology **156**, 585-92.

Montminy M, Brindle P, Arias J, Ferreri K, Armstrong R (1996) Regulation of somatostatin gene transcription by cyclic adenosine monophosphate. Metabolism **45**, 4-7.

Moore T and Haig D (1991) Genomic imprinting in mammalian development: a parental tug-of-war. Trends in Genetics **7**, 45-9.

Morgan HD, Santos F, Green K, Dean W, Reik W (2005) Epigenetic reprogramming in mammals. Human Molecular Cytogenetics **14** Spec No 1:R47-58.

Muller EE, Locatelli V, Cocchi D (1999) Neuroendocrine Control of Growth Hormone Secretion. Physiology Review **79**, 511-607.

Mutsuga N, Iwasaki Y, Morishita M, Nomura A, Yamamori E, Yoshida M, Asai M, Ozaki N, Kambe F, Seo H, Oiso Y, Saito H (2001) Homeobox protein Gsh-1-dependent regulation of the rat GHRH gene promoter. Molecular Endocrinology **15**, 2149-56.

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature **393**, 386-9.

Ohlsson R, Renkawitz R, Lobanenkov V (2001) CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends in Genetics **17**, 520-7.

Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J (2000) Active demethylation of the paternal genome in the mouse zygote. Current Biology **10**, 475-8.

Panning B, Jaenisch R (1996) DNA hypomethylation can activate Xist expression and silence X-linked genes. Genes and Development **10**, 1991-2002.

Peterson CL and Laniel MA (2004) Histones and histone modifications. Current Biology **14**, R546-51.

Plass C, Shibata H, Kalcheva I, Mullins L, Kotelevtseva N, Mullins J, Kato R, Sasaki H, Hirotsune S, Okazaki Y, Held WA, Hayashizaki Y, Chapman VM (1996) Identification of Grf1 on mouse chromosome 9 as an imprinted gene by RLGS-M. Nature Genetics **14**, 106-9.

Pombo CA, Zalvide J, Gaylinn BD, Dieguez C (2000) Growth Hormone-Releasing Hormone Stimulates Mitogen-Activated Kinase. Endocrinology **141**, 2113-2119.

Queller DC (2003) Theory of genomic imprinting conflict in social insects. BMC Evolutionary Biology **3**:15.

Rai K, Huggins IJ, James SR, Karpf AR, Jones DA (2008) Demethylation in zebrafish involves the coupling of a deaminase, a glycosylase, and Gadd 45. Cell **135**, 1201-1212.

Rakyan VK, Chong S, Champ ME, Cuthbert PC, Morgan HD, Luu KV, Whitelaw E (2003) Transgenerational inheritance of epigenetic states at the murine Axin (Fu) allele occurs after maternal and paternal transmission. PNAS **100**, 2538-43.

Reik W (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. Nature **447**, 425-32.

Reik W and Walter J (2001) Genomic imprinting: parental influence on the genome. Nature Reviews Genetics **2**, 21-32.

Renthal W, Nestler EJ (2009) Chromatin regulation in drug addiction and depression. Dialogues in Clinical Neuroscience **11**, 257-68.

Robertson KD (2005) DNA methylation and human disease. Nature Reviews Genetics **6**, 597-610.

Robinson KN, Manto K, Buchsbaum RJ, Macdonald JI, Meakin SO (2005) Neurotrophin-dependent tyrosine phosphorylation of Ras guanine-releasing factor 1 and associated neurite outgrowth is dependent on the HIKE domain of TrkA. Journal of Biological Chemistry **280**, 225-35.

Roth TL, Lubin FD, Sodhi M, Kleinman JE (2009) Epigenetic mechanisms in schizophrenia. Biochim Biophys Acta **1790**, 869-77.

Russo VEA, Martienssen RA, and Riggs AD (1996) Epigenetic mechanisms of gene regulation (Plainview, NY, Cold Spring Harbor Laboratory Press)

Santos F and Dean W (2004) Epigenetic reprogramming during early development in mammals. Reproduction **127**, 643-51.

Schulze SR and Wallrath LL (2007) Gene regulation by chromatin structure: paradigms established in Drosophila melanogaster. Annual Review of Entomology 2007 **52**, 171-92.

Scully KM, Jacobson EM, Jepsen K, Lunyak V, Viadiu H, Carriere C, Rose DW, Hooshman F, Aggarwal, AK, Rosenfeld MG (2000) Allosteric effects of pit-1 DNA sites on long-term repression in cell type specification. Science **290**, 1127-1131.

Shou C, Farnsworth CL, Neel BG, Feig LA (1992) Molecular cloning of cDNAs encoding a guanine-nucleotide-releasing factor for Ras p21. Nature **358**, 351-4.

Shou C, Wurmser A, Suen SL, Barbacid M, Feig LA, Ling K (1995) Differential response of the Ras exchange factor, Ras-GRF to tyrosine kinase and G protein mediated signals. Oncogene **10**, 1887-93.

Sleutels F, Zwart R, Barlow DP (2002) The non-coding Air RNA is required for silencing autosomal imprinted genes. Nature **415**, 810-3.

Slotkin RK & Martienssen R (2007) Transposable elements and the epigenetic regulation of the genome. Nature Reviews Genetics **8**, 272-285.

Smith FM, Garfield AS, and Ward A (2006) Regulation of growth and metabolism by imprinted genes. Cytogenetics Genome Research **113**, 279-91.

Stoger R, Kubicka P, Liu CG, Kafri T, Razin A, Cedar H, Barlow DP (1993) Maternal-specific methylation of the imprinted mouse Igf2r locus identifies the expressed locus as carrying the imprinting signal. Cell **73**, 61-71.

Sturani E, Abbondio A, Branduardi P, Ferrari C, Zippel R, Martegani E, Vanoni M, Denis-Donini S (1997) The Ras guanine nucleotide exchange factor CDC25Mm is present at the synaptic junction. Experimental Cell Research **235**, 117-23.

Suzuki S, Renfree MB, Pask AJ, Shaw G, Kobayashi S, Kohda T, Kaneko-Ishino T, Ishino F (2005) Genomic imprinting of IGF2, p57(KIP2) and PEG1/MEST in a marsupial, the tammar wallaby. Mechanisms of Development **122**, 213-22.

Thakur N, Tiwari VK, Thomassin H, Pandey RR, Kanduri M, Gondor A, Grange T, Ohlsson R, Kanduri C (2004) An antisense RNA regulates the bidirectional silencing property of the Kcnq1 imprinting control region. Molecular and Cell Biology **24**, 7855-62.

Tian X and Feig LA (2001) Basis for signaling specificity difference between Sos and Ras-GRF guanine nucleotide exchange factors. Journal of Biological Chemistry **276**, 47248-56.

Tian X and Feig LA (2006) Age-Dependent Participation of Ras-GRF Proteins in Coupling Calcium-Permeable AMPA-type Glutamate Receptors to Ras/Erk Signaling in Cortical Neurons. JBC **281**, 7578-82.

Tian X, Gotoh T, Tsuji K, Lo EH, Huang S, Feig LA (2004) Developmentally regulated role for Ras-GRFs in coupling NMDA glutamate receptors to Ras, Erk, and CREB. The EMBO Journal **23**, 1567-75.

Tonini R, Franceschetti S, Parolaro D, Sala M, Mancinelli E, Tininini S, Brusetti R, Sancini G, Brambilla R, Martegani E, Sturani E, Zippel R (2001) Involvement of CDC25mM/Ras-GRF1-dependent signaling in the control of neuronal excitability. Molecular and Cellular Neuroscience **18**, 691-701.

Ubeda F, and Wilkins JF (2008) Imprinted genes and human disease: an evolutionary perspective. Adv Exp Med Biol **626**, 101-15.

Urdinguio RG, Sanchez-Mut JV, Esteller M (2009) Epigenetic mechanisms in neurological diseases: genes, syndromes, and therapies. Lancet Neurology **8**, 1056-72.

Vadlamudi S, Kalhan SC, Patel MS (1995) Persistence of metabolic consequences in the progeny of rats fed a HC formula in their early postnatal life. American Journal of Physiology **269**, E731-8.

Varmuza S &Mann M (1994) Genomic imprinting – diffusing the ovarian time bomb. Trends in Genetics **10**, 118-123.

Vongs A, Kakutani T, Martienssen RA, Richards EJ (1993) Arabidopsis thaliana DNA methylation mutants. Science **260**, 1926-8.

Waddington, CH. (1942) The epigenotype, Vol 1 (London)

Wagschal A and Feil R (2006) Genomic imprinting in the placenta. Cytogenetic Genome Research **113**, 90-8.

Wang Y, Joh K, Masuko S, Yatsuki H, Soejima H, Nabetani A, Beechey CV, Okinami S, Mukai T (2004) The mouse Murr1 gene is imprinted in the adult brain, presumably due to transcription interference the antisense-oriented U2af1-rs1 gene. Molecular and Cell Biology **24**, 270-9.

Waterland RA and Jirtle (2003) Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Molecular and Cell Biology **23**, 5293-300.

Waterland RA, Dolinoy DC, Lin JR, Smith CA, Shi X, Tahiliani KG(2006) Maternal methyl supplements increase offspring DNA methylation at Axis Fused. Genesis **44**, 401-6.

Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ (2004) Epigenetic programming by maternal behavior. Nature Neuroscience **7**, 847-54.

Weaver IC, Champagne FA, Brown SE, Dymov S, Sharma S, Meaney MJ, Szyf M (2005) Reversal of maternal programming of stress response in adult offspring through methyl supplementation: altering epigenetic marking later in life. Journal of Neuroscience **25**, 11045-54.

Weidman JR, Murphy SK, Nolan CM, Dietrich FS, Jirtle RL (2004) Phylogenetic footprint analysis of IGF2 in extant mammals. Genome Research **14**, 1726-32.

Wilkins JF and Haig D (2003) What good is genomic imprinting: the function of parent-specific gene expression. Nature Reviews Genetics **4**, 359-68

Wolf B & Hager R (2006) A maternal-offspring coadaptation theory for the evolution of genomic imprinting. PloS Biology **4**, e380.

Wutz A, Smrzka OW, Schweifer N, Schellander K, Wagner EF, Barlow DP (1997) Imprinted expression of the Igf2r gene depends on an intronic CpG island. Nature **389**, 745-9.

Yakar S, Liu JL, Stannard B, Accili D, Sauer B, LeRoith D (1999) Normal growth and development in the absence of hepatic insulin-like growth factor 1. Proceedings of the National Academy of Sciences **96**, 7324-9.

Yamasaki K, Joh K, Ohta T, Masuzaki H, Ishimaru T, Mukai T, Niikawa N, Ogawa M, Wagstaff J, Kishino T (2003) Neurons but not glial cells show reciprocal imprinting of sense and antisense transcripts of Ube3a. Human Molecular Genetics **12**, 837-47.

Yamasaki Y, Kayashima T, Soejima H, Kinoshita A, Yoshiura K, Matsumoto N, Ohta T, Urano T, Masuzaki H, Ishimaru T, Mukai T, Niikawa N, Kishino T (2005) Tissue-specific relaxation of Igf2r imprinting is associated with neuron-specific histone modifications and lack of its antisense transcript Air. Human Molecular Genetics **14**, 2511-20.

Yang H and Mattingly RR (2006) The Ras-GRF1 exchange factor coordinates activiation of H-Ras and Rac1 to control neuronal morphology. Molecular Biology of the Cell **17**, 2177-89.

Yang H, Cooley D, Legakis JE, Ge Q, Andrade R, Mattingly RR (2003) Phosphorylation of the Ras-GRF1 exchange factor at Ser916/898 reveals activation of Ras signaling in the cerebral cortex. Journal of Biological Chemistry **278**, 13278-85.

Yehuda R, Bierer LM (2009) The relevance of epigenetics to PTSD: Implications for the DSM-V. Journal of Trauma Research, Epub Oct 7.

Yoon B, Herman H, Hu B, Park YJ, Lindroth A, Bell A, West AG, Chang Y, Stablewski A, Piel JC, Loukinov DI, Lobanenkov VV, Soloway PD (2005) Rasgrf1 imprinting is regulated by a CTCF-dependent methylation-sensitive enhancer blocker. Molecular and Cell Biology **25**, 11184-90.

Yoon BJ, Herman H, Sikora A, Smith LT, Plass C, and Soloway PD (2002) Regulation of DNA methylation of Rasgrf1. Nature Genetics **30**, 92-6.

Zhou Y, Xu BC, Maheshwari HG, He L, Reed M, Lozykowski M, Okada S, Cataldo L, Coschigamo K, Wagner TE, Baumann G, Kopchick JJ (1997) A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). PNAS **94**, 13215-13220.

Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S (2007) Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. Nature Genetics **39**, 61-9.

Zippel R, Balestrini M, Lomazzi M, Sturani E (2000) Calcium and calmodulin are essential for Ras-GRF1-mediated activation of the Ras pathway by lysophosphatidic acid. Experimental Cellular Research **258**, 403-8.

Zippel R, Gnesutta N, Matus-Leibovitch N, Mancinelli E, Saya D, Vogel Z, Sturani E (1997) Ras-GRF, the activator of Ras, is expressed preferentially in mature neurons of the central nervous system. Brain Research. Molecular Brain Research **48**, 140-4.